



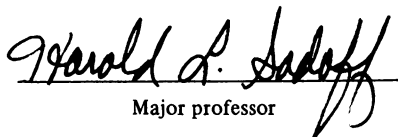
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PRODUCTION, ISOLATION AND IDENTIFICATION OF
UNIQUE LIPIDS BY AZOTOBACTER VINELANDII

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PRODUCTION, ISOLATION AND IDENTIFICATION OF
UNIQUE LIPIDS BY AZOTOBACTER VINELANDII

By

Chung-Jey Su

A DISSERTATION

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ABSTRACT

PRODUCTION, ISOLATION AND IDENTIFICATION OF UNIQUE LIPIDS BY AZOTOBACTER VINELANDII

By

Chung-Jey Su

Encystment in Azotobacter vinelandii can be induced by transferring exponentially growing cells from glucose to β -hydroxybutyrate (BHB) as the carbon and energy source in the medium. Because of its specificity as an inducer, the metabolism of BHB during the encystment was studied. Using ^{14}C -labeled BHB, two-thirds of the radioactivity incorporated into five-day old cysts was recovered in the lipid fraction. Thin layer chromatographic (TLC) analysis of cyst lipids revealed at least nine diazoreagent-reacting compounds. These were designated as AR_3 , AR_5 , AR_1 , AR_4 , AP_1 , AP_2 , AP_3 , AP_4 and AR_2 according to their motility on the chromatogram.

Beside AR_1 and AR_2 , which have already been identified as mixture of 5-n-heneicosylresorcinol, 5-n-tricosylresorcinol and their galactose derivatives, AR_3 was identified as mixture of 6-n-heneicosylresorcylic acid methyl ester and 6-n-tricosylresorcylic acid methyl ester. AR_5 was identified as mixture of 5-n-heneicosyl-4-acetyl-resorcinol

and 5-n-tricosyl-4-acetyl-resorcinol. AR₄ was identified as mixture of 5-n-(2-hydroxy)-heneicosylresorcinol and 5-n-(2-hydroxy)-tricosylresorcinol, AP₁ was identified as mixture of 6-n-heneicosyl-4-hydroxy-pyran-2-one and 6-n-tricosyl-4-hydroxy-pyran-2-one and AP₂ identified as mixture of 6-(2-oxotricosyl)-4-hydroxy-pyran-2-one and 6-(2-oxopentacosyl)-4-hydroxy-pyran-2-one. AP₃ and AP₄ were not identified, but their reactions to various spray reagents were similar to AP₁ and AP₂. These nine components account for 81% of the total radioactivity in the lipid fraction of mature cysts.

The carbon atoms in these compounds were established to be mostly derived from BHB used to induce encystment. The biosynthesis of these compounds during encystment were studied by inducing with ¹⁴C-BHB, extracting the lipids, separating them by TLC and then determining the radioactivity in each compound. The synthesis of these unique lipids started at 8-12 hours after the induction of encystment and reached a plateau 2 days after the induction of encystment. After their formation, these compounds remained relatively stable for the rest of encystment period. Most of the phenolic compounds (AR₁, AR₂, AR₃ and AR₄) remained relatively stable during the germination but the pyronic compounds (AP₁ and AP₂) were degraded during the germination.

About 70% of these unique lipids were located in the cyst central body, 23% in the exine and 7% in the intine. The phenolic compounds were evenly distributed in exine,



intine and central body according to the proportion of lipid distribution in these three fractions but the pyronic compounds were concentrated in central body fraction.

The physiological significance of these compounds for the cysts is unknown but they may function as structural components. Their biosynthesis pathways are presumed to be via polyketides like similar natural products.



Dedicated to

my parents, my wife and my daughter.

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INTRODUCTION

Azotobacter vinelandii are gram negative, free-living, nitrogen-fixing bacteria which inhabit the soil (53). Exponentially growing cells appear as large ovoid rods under the microscope. At the end of exponential growth, a small percentage of cells undergo differentiation which results in the formation of cysts (101). Cysts are metabolically dormant and are more resistant to deleterious physical conditions than vegetative cells (83). Under suitable environmental conditions, they can germinate readily and convert back into vegetative cells (54) and thus complete the life cycle of A. vinelandii.

Encystment can be induced by transferring exponentially growing vegetative cells from glucose to 0.2% β -hydroxybutyrate (BHB) (51). The entire encystment process is completed in 4-6 days and results in the conversion of more than 90% of the vegetative cells into cysts (51). BHB serves both as carbon source and energy source during the encystment (35).

Like other kinds of cell differentiation processes, encystment involves a programmed activation of some particular genes and turn-off of some other genes during its

course. BHB is a specific inducer of encystment whereas other compounds such as amino acids, carbohydrates and short chain fatty acids such as acetate or propionate are without effect. n-butanol and crotonic acid do induce encystment but probably exert their effect by being first converted into BHB or its metabolic product by bacteria (51). An understanding of how BHB functions to trigger the whole encystment process is critical to our understanding of cellular differentiation in this organism.

BHB is a normal metabolite in Azotobacter and these cells accumulate poly- β -hydroxybutyrate (PHB) as an energy reserve during vegetative growth (87). PHB is synthesized and degraded via acetyl CoA (21), a key intermediate in lipid metabolism and TCA cycle. Using ^{14}C -BHB labeled at the C-3 position to study the BHB metabolism during encystment, two-thirds of the total radioactivity incorporated into the cysts can be recovered in lipid fraction after 5 days (75). Thus, the importance of lipid metabolism during encystment cannot be overemphasized. Lin and Sadoff (49) found cysts contain twice as much lipid as vegetative cells on the dry weight basis. A wide variety of fatty acids were found in the cysts whereas only four major fatty acids are present in vegetative cells (39). Cis-9,10-methylene hexadecanoic and lactobacillic acids are two unique fatty acids synthesized during encystment (77). These compounds can be detected as early as 6 hours after the induction of encystment and are synthesized at the expense of palmitoleic



and cis-vaccenic acids by the addition of a methylene group across a carbon-carbon double bond (104). They eventually account for 25% of total fatty acids in mature cysts.

In a recent investigation of cyst lipids, Reusch and Sadoff (75) fractionated the cyst lipids into neutral, "glyco-" and phospholipids on a silicic acid column (45). They found that 82% of lipids in mature cysts were in the acetone or "glycolipid" fraction and most of lipids in this fraction were derived from BHB used to induce encystment. Further analysis of this fraction by thin layer chromatography revealed two major molecular species where each species consisted of two homologues. The four compounds were identified as 5-n-heneicosylresorcinol, 5-n-tricosylresorcinol and their galactoside derivatives. In my research, I have isolated several new compounds in the acetone fraction (glycolipid) and established their structure by ultraviolet (UV), infrared (IR), nuclear magnetic resonance (NMR) and mass spectroscopy. The time course for the appearance of these lipids was also established in encysting cells. The origins and possible roles of these compounds is the subject of the Discussion section of this dissertation.

The dissertation is organized into the following sections: an Abstract of the dissertation; Introduction; a Literature Review which contains information about the physiological properties of the organism, studies of encystment and metabolic patterns in A. vinelandii with



special reference to β -hydroxybutyrate; Materials and Methods utilized in the research; and Results and Discussion of the research. An Appendix is included which contains a description of unpublished research results.



LITERATURE REVIEW

A. Physiology of Azotobacter vinelandii

A. vinelandii are gram negative, free-living, nitrogen-fixing bacteria originally isolated by Lipman in 1903 from a soil sample from Vineland, New Jersey (53). Exponentially growing cells of A. vinelandii appear as large ovoid rods, about 2 x 4 um in size, frequently in pairs under microscope. The organism produces large amounts of extracellular polysaccharide to give its colony a mucoid appearance on agar surfaces. Fresh cultures of A. vinelandii are motile due to the presence of flagella (37), but the motility is lost gradually as the culture approaches the end of exponential growth. Cells accumulate poly- β -hydroxybutyric acid apparently as an energy reserve. The morphology of A. vinelandii varies with growth conditions and as many as five morphological forms have been described (38). Azotobacter is widely distributed in the soil with pH between 6-10 (38). In the laboratory, it can be easily grown in synthetic medium in which potassium, magnesium, calcium, iron, sulfate and phosphate are essential mineral nutrients (96). When fixed nitrogen is not available in the medium and cells have to fix atmosphere nitrogen, then



molybdenum is also essential for its growth because this element is one of the components of nitrogenase (14).

Alcohols such as ethanol, propanol, butanol and glycerol, organic acids such as acetate, propionate, butyrate and succinate and carbohydrate such as glucose, sucrose, fructose, galactose and maltose all can be used as both carbon and energy source by this organism (38).

Azotobacter is a strict aerobe with a high respiration rate (52). Carbohydrates such as glucose are metabolized through the Entner-Doudoroff pathway into 2 pyruvate with the generation of 1 adenosine triphosphate (ATP) and 2 NADH (62, 85). Pyruvate is further oxidized into CO_2 and H_2O in the tricarboxylic acid (TCA) cycle (85) with the generation of metabolic intermediates and ATP. However, an excess of O_2 inhibits the growth of Azotobacter by inhibiting nitrogen fixation which requires a reducing condition (103).

Although Azotobacter grows well in simple synthetic medium, it does not grow well in nutrient broth (38) because its utilization of organic nitrogen is very poor (30). Some amino acids (e.g., glycine and leucine) inhibit the growth of Azotobacter at a concentration of 0.1% (24). On the other hand, ammonium ion, nitrate and urea can all serve as nitrogen sources for Azotobacter. When fixed nitrogen is not available, Azotobacter can fix atmospheric nitrogen under aerobic growth conditions. Nitrate and urea are reduced or degraded into ammonium ion and, when present in high concentration, are assimilated into the cell



components via the glutamate dehydrogenase pathway (39, 96). When the ammonium ion concentration is low and cells have to fix nitrogen from the air, ammonia is assimilated into the cell via the enzymes glutamine synthetase and glutamate synthetase (25, 40).

During nitrogen fixation, N_2 is reduced to $2 NH_3$ by nitrogenase using reduced nicotinamide adenine dinucleotide phosphate (NADPH) as the reductant and ATP as the energy source (88). Nitrogenase is an enzyme complex consisting of 2 kinds of metal-containing proteins. In A. vinelandii, complex I (Mo-Fe protein) is an enzyme containing molybdenum and iron with a molecular weight of 245,000 dalton (89). Component II (Fe-protein) is a component I reductase which contains iron and has a molecular weight of 60,000 daltons (90). Both proteins are needed for the enzymatic activity and a ratio of 1:1 is optimal for the highest activity. A third protein, iron-sulphur protein II, with a molecular weight of 24,000 dalton is also associated with nitrogenase in Azotobacter and may have a regulatory role (79) or contribute to the stability of the Azotobacter nitrogenase in the presence of oxygen (32).

A. vinelandii DNA has a G + C content of 63 to 66% (56). Each cell in early exponential growth phase contains about 15×10^{-14} g of DNA which is equivalent to 10 times that of E. coli (78). Possible DNA redundancy in the Azotobacter gene might explain the difficulty encountered in isolating mutants from this organism.



Recently, Page and Sadoff (67, 68) have developed a method for the transformation of Azotobacter. A. vinelandii becomes competent for a period of time approaching the end of exponential growth. Transformation competence decreases rapidly as cells enter stationary phase. Optimal transformation occurs at 30°C at a pH of 7.0-7.1. Magnesium and phosphate are essential for the transformation. High concentrations of calcium in the medium enhance the production of capsule and hence are inhibitory to the transformation (70). Ammonium ion in the iron-free medium increases the transformation frequency. Only NH_4^+ , NO_3^- or urea can serve as nitrogen sources for growth and, at the same time, allow the induction of competence. Other nitrogen sources which do not inhibit glutamine synthetase inhibit the induction of competence (70). Glucose, sucrose, manitol and glycerol all serve as good carbon sources in the induction of competence but not acetate or β -hydroxybutyrate. Competence also can be increased 1000-fold in early exponentially growing cells by the addition of 1mM c-AMP (68). A. vinelandii can also be transformed with Rhizobium spp. DNA (10, 66).

B. Cysts of Azotobacter vinelandii

1. Induction of encystment and germination

Among the four genera of Azotobacteriaceae, only Azotobacter sp. are able to form cysts (3). Cysts are metabolically dormant cells that are more resistant than



vegetative cells to radiation, sonic treatment, heat and dessication (83). Thus, cysts can survive under unfavorable conditions where the survival of vegetative cells is impossible. Encystment occurs at the end of exponential growth when one or more of the growth factors become limiting. At this time, vegetative cells start to accumulate poly- β -hydroxybutyrate (PHB) and some cells eventually differentiate into cysts (98, 101). The extent of encystment in a culture is related to the intracellular level of PHB and normally less than 0.1% of the total population become cysts (86) in cultures grown on a carbohydrate such as glucose. Encystment also can be induced by growing cells in n-butanol (100) or by shifting the exponentially growing vegetative cells from glucose to 0.2% BHB as carbon source (51). This latter two-step encystment procedure developed by Lin and Sadoff (51) results in a more than 90% yield of cysts after 5 days of incubation. If the vegetative cells are not washed free of glucose before the addition of BHB, abortive encystment occurs resulting in a low yield of cysts and release of a viscous polymer into the medium (51).

The BHB used to induce encystment is absorbed and metabolized by the cells (35). Tracer studies with ^{14}C -3-BHB showed that about 10% of the BHB in the medium was released as CO_2 and about 25% was incorporated into cyst materials. Radioactive BHB was incorporated into the RNA, protein and lipid fractions (35). The radioactivity in the RNA fraction increased steadily, reached a peak at 9 hours



after the induction and then decreased slowly. The radioactivity in the protein fraction increased steadily up to 9 hours and continued thereafter at a lower rate. The radioactivity of the lipid fraction increased slowly up to 9 hours, then sharply up to 24 hours and then leveled off. No radioactive BHB was incorporated into the DNA fraction.

When metabolizable carbon sources become available in the cyst environment and given suitable pH, temperature, moisture, minerals and carbon source, cysts germinate and grow into vegetative cells. Germination can be induced by suspending cysts in Burk's N-free medium plus glucose, sucrose or acetate as the carbon source (48). The whole process takes about 12 hours and can be roughly divided into 3 stages of 4 hours each: Germination, outgrowth and transition (48).

2. Morphological changes during encystment and germination

The morphological changes during the encystment and germination of Azotobacter cysts have been studied microscopically (78, 98) and electron microscopically (36, 48, 84, 100). When viewed by phase contrast microscopy, vegetative cells are large rods, actively motile, and in many stages of division. The cytoplasm is quite homogeneous and not refractile, although old cells do contain refractile granules. When examined under the electron microscope, the cells ribosomes are seen as dark granules dispersed throughout the cytoplasm. DNA can be seen in the electron

transparent areas of cytoplasm and membranous vesicles can be seen within the cytoplasm and along the periphery of cell. Surrounding the cells is a double layered cytoplasmic membrane and a triple layered cell wall. Extrusions protruding from the cell surface also can be observed (36).

Microscopically, mature cysts appear as round, refractile and non-motile cells. Thin sections, viewed in the electron microscope, show cysts to be made up of three major components; (a) an electron dense central body containing some PHB granules surrounded by (b) a less dense intine and (c) a multilayered exine (36, 50, 73).

Upon the induction of encystment, cells undergo a last round of cell division (78) which takes about 4 hours. During this period, cells start to "round up" and accumulate PHB. As encystment progresses, each cell is gradually surrounded by a layer of capsular material and a number of protrusions or "blebs" are excreted from the cell surface. These aggregate and flatten into sheets which ultimately become the exine. After 36 hours, the exine, intine and central body can be easily seen but approximately 80 more hours are required for the completion of intine and exine structure. As the cysts mature, the central body becomes rather compact (36).

Upon the induction of germination, the cysts lose their refractility gradually over a 4 hour period (54). In the first 2 hours after induction, intines become more electron transparent, a fibrillar structure around central

body can be observed, but little change occurs in exine and central body. As the germination progresses, the intine becomes less electron dense and the vesicular and fibrillar structure of intine can be easily observed. Four hours after germination commences, the PHB in the central body starts to be metabolized. The central body starts to elongate at 6 hours signalling the beginning of outgrowth. The central body continues to grow and starts to divide, and at 8 hours it emerges from the disintegrating exine and becomes a young vegetative cell. According to Lin et al. (48) the young vegetative cells which emerge are still more resistant to sonication than exponentially growing vegetative cells and it takes another 4 hours of transitional period for the outgrown cells to become normal vegetative cells (48).

3. Metabolic changes during encystment and germination

When glucose-grown vegetative cells of A. vinelandii are induced to encyst with BHB, they must shift from carbohydrate to lipid metabolism. A readjustment of metabolic rate and induction of new metabolic pathways is necessary to cope with this kind of change. Some enzymes of the Entner-Doudoroff pathway are not required for the metabolism of BHB, but at the same time, glyoxylate and gluconeogenesis pathway enzymes must be induced for the continuous operation of TCA cycle and to provide carbohydrate for the synthesis of certain cyst components.



A number of the metabolic events which occur during encystment of A. vinelandii have been studied (35, 78). The initiation of DNA synthesis stops immediately and no synthesis can be detected at 4 hours after induction of encysts (35). The DNA content per cell drops during the course of encystment from 15×10^{-14} to 3.4×10^{-14} g (78). The nitrogen fixing ability of cells also drops sharply and losses of 30% of the original activity occur within 15 minutes after the induction of encystment with complete loss after 1 hour (35). RNA synthesis continues at a reduced rate until 12 hours into encystment. Protein synthesis during encystment occurs at about one-third the rate at which it occurs in vegetative cells (35) and continues throughout the entire encystment period. Because of the early cessation of nitrogen fixation during the encystment of A. vinelandii, macromolecular synthesis must depend on the turnover of existing protein and RNA. The specific activity of glucose-6-phosphate dehydrogenase, which is a key enzyme in glucose degradation, decreases steadily after the induction of encystment and is almost completely gone after 9 hours. At the same time. BHB dehydrogenase, the first enzyme in BHB metabolism, is being synthesized. BHB dehydrogenase reaches a peak activity at 6 hours after induction of encystment, decreases slightly, then increases to another peak at 21 hours. Isocitrate dehydrogenase activity follow the same pattern as BHB dehydrogenase. The two glyoxylate shunt enzymes, isocitrate lyase and malate

synthetase, are derepressed immediately after induction of encystment, reach a peak at 3 hours, decrease to a low value by 9 hours, then increase again at 15 hours to reach a high level during the late encystment. Aldolase and fructose diphosphatase (FDPase), two key enzymes in the gluconeogenesis pathway, also have a temporal relationship during encystment. Aldolase reaches a maximum at 6 hours, decreases to the original value at 18 hours and then increases to another peak at 24 hours. FDPase activity increases and reaches a maximum at 9 hours. After that, it decreases to a low point at 18 hours and then increases to another peak of activity at 27 hours.

Cysts in Burk's buffer at 30°C start to germinate upon the addition of glucose. The germination and outgrowth phases each have their characteristic rates of O_2 consumption, CO_2 evolution and RNA and protein synthesis (54). The O_2 uptake and CO_2 evolution begin very soon after the addition of glucose and constitute the first signs of germination. Both the O_2 uptake and CO_2 evolution start at low rates initially and increase to constant values at about 4 hours (54). RNA synthesis occurs within 20 minutes after the initiation of germination and proceeds at a low rate up to 3.5 hours. It then increases sevenfold by 5 hours post-induction and remains at this rate throughout outgrowth. Protein synthesis occurs very soon after the initiation of germination and proceeds at a constant rate up to 5 hours. It then increases 3 fold and remains at that rate until the

completion of outgrowth (28). Both DNA synthesis and N_2 fixation start at about 5 hours after induction signalling the beginning of the outgrowth (54).

4. Compositional differences between vegetative cells and cysts of *Azotobacter vinelandii*

Vegetative cells of *A. vinelandii* contain 28% carbohydrate, 52% protein, 9.2% lipid and 7.1% ash by dry weight. By comparison, cysts contain 45% carbohydrate, 26% protein, 16% lipid and 8.8% ash (49). Cysts have been further fractionated into exine, intine and central body (50). Exines were prepared by rupturing the cysts in Tris (hydroxymethyl)aminomethane (Tris) buffer, pH 7.8 plus 3 mM of ethylenediaminetetraacetic acid and isolated by differential and isopycnic centrifugation. Intines were soluble in the buffer and were prepared by rupture of cysts in the Tris buffer plus EDTA, collecting the supernatant fluid after centrifugation and then dialyzing it against distilled water to remove small molecules. An analysis of the purified exines and intines indicates that exine contains 44% of carbohydrate, 9.1% protein, 36% lipid and 4.1% ash whereas intine contains 32% carbohydrate, 28% protein, 28% lipid and 3.2% ash (49).

The slime of *Azotobacter* vegetative cells contains mostly partially acetylated polyuronic acids (D-mannuronic and L-guluronic acids) plus small amounts of glucose and rhamnose (18, 46). The two uronic acids are distributed along the polymer chain in the typical block wise fashion

characteristic of alginates from brown algae (46). The ratio of the uronic acids in the slime depends on the calcium concentration in the medium. High calcium concentrations result in the production of slime rich in L-guluronic acid. An enzyme "5-epimerase" capable of converting D-mannuronic into L-guluronic has been demonstrated in medium in which A. vinelandii has grown (33). The activity of the epimerase depends on the calcium concentration of the medium.

Uronic acid accounts for 40% and 72% of the carbohydrate in the exine and intine, respectively, of Azotobacter cysts (69). D-Mannuronic and L-guluronic acids are the only two uronic acids present. The exine is rich in polyguluronic acid and the intine is rich in polymannuronic acid (69). This may be an effect of the high concentration of calcium at the beginning of encystment when the exine is formed. Calcium enhances the 5-epimerase activity and converts the D-mannuronic into L-guluronic acid. The resulting polymers bind calcium ions selectively (82) and help form the sheet like structures of exine. The completed exine contains a relatively large amount of calcium and forms a physical barrier which prevents the influx of calcium ions.

Exponentially growing vegetative cells of A. vinelandii contain about 10% free lipids and an additional 2% bound lipids (39, 49). About 80% of the free lipids are phospholipids of which 64% is phosphatidylethanolamine, 27%



is phosphatidylglycerol, plus minor amounts of phosphatidyl-N-methyl-ethanolamine, phosphatidylcholine and cardiolipin (74). In stationary phase cells, cardiolipin increases to 23% of the total phospholipid while phosphatidylglycerol content decreases to 13% of the total phospholipid (74). Major fatty acids in the vegetative cells are myristic acid (7%), palmitic acid (35%), palmitoleic acid (41%) and octadecenoic acid (17%) (39). Cysts contain twice as much lipid as vegetative cells. A wide variety of fatty acids have been identified in the cysts (49, 77). Among these, 2 cyclopropane fatty acids, cis-9,10-methylenehexadecanoic and lactobacillic acid, are unique in Azotobacter cysts (77). They are synthesized at the expense of palmitoleic acid and octadecenoic acid after induction of encystment (77) and appear as early as 6 hours after induction of encystment. Eventually each of the cyclopropane fatty acids reaches 10% of the total fatty acids in the mature cysts.

C. Poly- β -hydroxybutyrate (PHB) Metabolism in Azotobacter

BHB is a normal metabolite in cells which can be synthesized by the condensation of 2 acetyl CoA into acetoacetate followed by reduction to BHB. BHB is also a specific inducer of encystment of A. vinelandii. The procedure used to induce encystment probably results in a sudden rise of the intracellular BHB concentration. How such a concentration increase of a normal metabolite can trigger the sequence of events resulting in the formation

of dormant cysts is an important question. A complete understanding of BHB metabolism in the cell help us to answer this question.

A wide variety of bacteria accumulate BHB as a polymer (PHB) intracellularly as an energy reserve when their carbon supply is plentiful (20). PHB is a straight chain homopolymer of D(-)-3-hydroxybutyrate. Natural PHB has a molecular weight between 60,000-250,000 daltons (55) and can be observed by light microscopy as refractile granules in bacteria or as dark bodies when stained by Sudan black (60). Under the electron microscope, a membranous structure can be observed surrounding the PHB granules (56). This coat can be removed by treating the native PHB with aqueous acetone (26) and has been found to consist of lipids and protein, the protein often possessing PHB polymerization activity (31).

PHB is an excellent storage form of carbon and energy sources because it is in a highly reduced state, virtually insoluble in water, and thus does not exert any osmotic effect. Macrae and Wilkinson (57) observed in Baccilus megaterium that, as the ratio of carbon and energy source to the nitrogen source of the medium was increased, the amount of PHB accumulated per cell also increased. Pyruvate, 3-hydroxybutyrate and glucose increase the yield of PHB in the same organism. When acetate was added together with any of these substrates the extent of PHB synthesis was greatly increased (58). In continuous culture experiments

with B. megaterium (94), it was observed that PHB accumulated when any of the nutrients in the culture become a limiting factor. Senior et al. (80) studied the accumulation of PHB in batch cultures of A. beijerinckii grown in nitrogen-free medium. They found that cells started to accumulate PHB at the end of their exponential growth and continued to do so during the stationary phase. The amount of PHB accumulated in the cell was proportional to the amount of glucose in the medium. Continuous culture studies revealed that neither nitrogen nor carbon source limitation in the culture led to the accumulation of large amounts of PHB but that oxygen limitation was the major factor in its accumulation in Azotobacter. PHB accumulation ceased when the O_2 limitation in the culture was relaxed. The possibility of using PHB as reducing power sink to regulate the redox potential in Azotobacter had been suggested (21).

The biosynthesis of PHB in A. beijerinckii is dependent on three enzymes (21). β -Ketothiolase catalyzes the condensation of 2 acetyl CoA into acetoacetyl CoA with the release of CoASH. Acetoacetyl CoA is then reduced into D(-)-3-hydroxybutyryl CoA by acetoacetyl CoA reductase. NADPH is the preferred coenzyme in this reaction. D(-)-3-hydroxybutyryl CoA is then incorporated into PHB by PHB synthetase which is usually associated with the PHB granules.

The degradation of PHB in this organism involves another pathway. PHB is degraded into BHB by a PHB depolymerase after which BHB is then oxidized into acetoacetate

by BHB dehydrogenase using NAD as the coenzyme. The enzyme "thiophorase" converts acetoacetate into acetoacetyl CoA using succinyl CoA as CoA donor. Acetoacetyl CoA is further cleaved into 2 acetyl CoA by the β -ketothiolase which catalyzes a reversible reaction.

The regulation of PHB metabolism in A. beijerinckii is shown in the following scheme (Fig. 1).

When O_2 becomes limiting in an Azotobacter culture, NADPH and NADH increase and inhibit citrate synthetase and isocitrate dehydrogenase activity. The inhibition of these two enzymes results in the accumulation of acetyl CoA and reduces the CoASH concentration in cells. This condition favors the formation of acetoacetyl CoA in the reaction catalyzed by β -ketothiolase and results in the synthesis of PHB. High concentrations of CoA inhibit the formation of acetoacetyl CoA. Acetoacetyl CoA inhibits the acetoacetyl CoA reductase (76). Only when the NADPH concentration is high and CoASH is low, (e.g.: during O_2 limitation) would acetoacetyl CoA reduction occur and PHB be synthesized. Simultaneously, high NADH inhibits the depolymerization of PHB by inhibiting the BHB dehydrogenase and thus preventing unrestricted cycling of PHB. When O_2 limitation is relaxed, the decreased concentration of NADPH and NADH will restrict the biosynthesis of PHB but not until the carbon source is also limited e.g., decreased pyruvate concentration. This will result in the decrease of acetyl CoA and increase of CoASH. β -Ketothiolase will then catalyze the degradation

(After Dawes E.A. and P.J. Seniopr) (21)

-----indicates inhibitor



of acetoacetyl CoA into acetyl CoA with subsequent PHB degradation.

Slepecky and Law (81) concluded that while PHB accumulation is not a prerequisite for spore formation in B. megaterium, the polymer serves as a source of carbon and energy during sporulation. Kominek and Halvorson (42) came to the similar conclusion in studies of sporulation in B. cereus. Stevenson and Socolofsky (86) studied cyst formation and PHB accumulation in Azotobacter and found that cells accumulated large amounts of PHB before the on-set of encystment. The accumulated PHB was subsequently degraded to provide carbon and energy for encystment. The percentage of encystment in a culture was directly proportional to the amount of PHB accumulated. Good carbon sources which stimulate PHB accumulation, e.g., butanol or glucose, also result in a higher percentage of encystment.

MATERIALS AND METHODS

Growth of Bacteria

A. vinelandii ATCC 12837 was used throughout this study. Cells were cultivated in Burk's nitrogen-free medium (97) with either 1% glucose or 1% sodium acetate as the carbon source. The culture was incubated at 30°C with shaking. Bacterial growth was monitored by measuring the absorbance at 620 nm (OD_{620}) with a spectrophotometer.

Induction of Encystment

Encystment was induced according to the method of Lin and Sadoff (51). Exponentially growing cells ($OD = 0.7$) were harvested by centrifugation, washed once with Burk's buffer (medium without carbon source) and suspended in the same volume of Burk's buffer. β -Hydroxybutyrate (BHB) was added to a final concentration of 0.2% and the culture was then incubated at 30°C with shaking. Mature cysts were formed by the fifth day after the shift to BHB. Cysts were harvested by centrifugation, washed once with Burk's buffer and stored at -20°C.

Germination of Cysts

The fate of unique cyst lipids was studied during germination. Sufficient cysts were suspended in 100 ml

sterile Burk's buffer to produce a final OD = 0.7 and incubated at 30°C. Germination was started by adding 2 ml of a sterile 50% solution of glucose to the cyst suspension and incubating at 30°C with shaking. Complete outgrowth of vegetative cells occurred by 8 hours after germination.

Lipid Extraction

Total lipids in the vegetative cells or cysts were extracted according to the method of Bligh and Dyer (11). Cells were harvested by centrifugation, washed once and then suspended in a small volume of water. To each 0.8 ml of cell suspension, 1 ml of chloroform and 2 ml of methanol were added. The mixture was shaken vigorously (vortexed) for 1 minute to form one phase and kept at 4°C overnight.

The cell residue was filtered out and the solution was separated into 2 phases by the addition of 1 ml of chloroform and 1 ml of H₂O to every 3.8 ml of solution. The biphasic system was shaken in a vortex mixer and then centrifuged briefly to again separate it into two layers. The chloroform layer was washed once with 0.7% NaCl solution, rinsed twice with distilled water and taken to dryness under a hood.

Removal of PHB

Occasionally, PHB is co-extracted with other lipids in the chloroform layer and interferes with the chromatographic separation of lipids. The removal of PHB was achieved by drying the chloroform layer slowly in a hood.

PHB forms a membrane-like sheet under these conditions (95) which does not go into solution when lipids other than PHB are dissolved in a chloroform:methanol (2:1) mixture.

Methylation of Samples

Lipids usually become more volatile and easier to separate in gas chromatograph columns after methylation. Methylation also can help to determine the number of "active exchangeable protons" in a molecule. Methylation was carried out with diazomethane. Diazomethane was prepared by distillation from an ethereal solution resulting when its precursor N-N-dinitroso-N,N-dimethylterephthalamide was mixed with 10% KOH. Enough diazomethane was added to the compounds undergoing methylation so that a yellow color persisted for at least 20 hours. Completeness of methylation was checked by thin layer chromatography (TLC).

Chromatographic Methods

A. Column chromatography

Primary separation of crude lipids extracted from cysts was carried out on florisil (Matheson Coleman and Bell, 60-100 mesh) in a 2.5 x 50 cm column. About 80 g of florisil was used for 2 g of crude lipid extract. The absorbant was suspended in chloroform, degassed under vacuum, and then packed into a column. Samples were dissolved in a small amount of hot chloroform and methanol mixture (2/1,v/v) and loaded onto the column. The column

was eluted in sequence with chloroform (200 ml), 20% acetone in chloroform (100 ml), 60% acetone in chloroform (50 ml), 100 ml acetone (50 ml), 25% methanol in chloroform (100 ml), 50% methanol in chloroform (150 ml) and methanol (100 ml). The flow rate was 0.5 ml/minute and 10 ml fractions of eluate were collected in test tubes. The solvent was evaporated under a stream of N₂ gas and the lipid in each fraction was dissolved in chloroform:methanol (2:1, v/v) and then analyzed by thin layer chromatography (TLC).

Fractions with similar composition were pooled and further fractionated on a silicic acid column (silic AR cc-7 Mallinckrodt Analytic Reagent). Silicic acid was activated at 120°C for 16 hours, cooled in a dessicator and then made into a slurry with chloroform. This was degassed under vacuum and then packed into a 1.3 x 25 cm column (1g/10 mg of sample). Samples were loaded onto the column in the same way as in the florisil columns. Elution of fractions was stepwise beginning with chloroform, then increasing concentrations of acetone in chloroform followed by increasing concentrations of methanol in chloroform. The volume of each solvent employed was approximately the volume of packed column bed at a flow rate of 0.3 ml/minute. Approximately 10 ml volumes of eluate were collected in test tubes and the lipids in each tube were analyzed by TLC.

B. Thin layer chromatography

Precoated 2000 μ silica gel G glass plates (Analtech Labs) were used for preparative separation of lipids. From 2 to 5 mg of sample was applied as a strip on each plate. Precoated silica gel 60 aluminum sheets (E.M. Labs.) were used for analytical separation. Development was in either solvent system - 1) Carbon tetrachloride : Acetone (90:10, v/v) or

2) Chloroform : acetone : Methanol
(12:4:1, v/v/v)

Total lipids were detected by exposure of plates to iodine vapor. Diazotized sulphanilamide reagent (43) was used to locate compounds that were of interest in this study. The vanillin test (43) was used to identify phenolic compounds. Preparative plates were dried under nitrogen after being developed. At this point, the plate was partially covered with a glass sheet leaving about a 2 cm strip at the edge to be sprayed with the appropriate reagent. After spraying, the remaining areas corresponding to the R_f of compounds of interest were scraped into individual flasks and extracted with chloroform : methanol (2:1, v/v). The solvent was evaporated under nitrogen and the residue analyzed by analytical TLC. The procedure was repeated until there was only one compound present as detected by analytical TLC.

Spray reagent : Diazotized sulfanilamide reagent :
3 g of sulfanilamide were dissolved in a solution of 200 ml H₂O, 6 ml of 36% HCl and 14 ml of n-butanol. 0.3 g of



sodium nitrite was added to 20 ml of this cold solution immediately before spraying chromatograms. Phenolic compounds appeared as red-orange areas immediately after spraying. Pyronic compounds reacted slower and turned yellow to orange color. The background became yellow several hours after spraying the chromatograms. Spraying with 10% of sodium carbonate prevented this but may have decreased the color intensity of some spots (pyrones).

Vanillin test : Samples on plates which had been sprayed with 1% vanillin in glacial acetic acid were dried. Plates were then sprayed with H_2SO_4 in ethanol (1 part of concentrated H_2SO_4 mixed with 4 parts of absolute alcohol) and heated at 100°C for 5 minutes. Phenolic compounds turned pink.

C. Gas chromatography

Gas chromatography (GC) was conducted in a Varian Aerograph 1440 gas chromatograph equipped with a flame ionization detector. Helium was used as the carrier gas at a flow rate of 30 cc/min. A 183 cm x 0.32 cm or 61 cm x 0.32 cm stainless steel column was used. Packing was 1.5% SE-30 on chrom G A/W, 100/120 mesh. Methylated derivatives of unknown lipids were chromatographed either isothermally or with temperature programming.

D. High pressure liquid chromatography (HPLC)

Because samples purified from preparative TLC were mixtures of 2 homologues of different chain length, some

samples were further separated by HPLC. These separations were achieved on a preparative reverse phase column (LiChrosorb RP-8, 5 μ particle size, packed in a 10 (ID) x 250 mm stainless steel column (Altex Associates Inc.)). The sample injector used was a Rheodyne model 7120 fitted with a 200 μ l loop. Pressure was applied with a Milton Roy model 396 minipump. Samples were detected with an Altex analytical model 153 UV-Vis detector at 280 nm connected to a Linear recorder model 255.

The mobile phase solvent was either 10% H₂O in methanol or 20% H₂O in methanol depending on the compounds being separated. About 500 ug of sample in chloroform: methanol (2:1) were applied to the column. The mobile phase was pumped at 2 ml/min, eluates were detected and each peak concentration of compound was collected in a flask. Samples were extracted from the flasks with chloroform.

Spectrometric Methods

A. Ultraviolet (UV)

UV absorption spectra were taken with a Beckman DU spectrophotometer model 2400 employing 95% ethanol as the solvent.

B. Infrared (IR)

IR spectra were taken as Nujol mulls or dilute solutions in chloroform or carbontetrachloride depending



on the solubility of the sample. A Perkin-Elmer 700 infra-red spectrophotometer was used.

C. Mass spectra

A Hewlett-Packard 5985 GC-MS system was used to take low resolution mass spectra (MS). A direct probe was used for non-volatile samples. Methylated samples were run in GC-MS combination. Electron impact was the mode of ionization with the ionization voltage at 70 eV. Electron multiplier voltage was at 2000 V. Methane was used in chemical ionization to obtain the molecular ions of unstable samples. Peak matching for precise ion masses were run in a Varian MAT CH-5DF mass spectrometer with a direct probe. The respective operating conditions were: accelerating voltage at 3 KV, ionization voltage at 70 eV and electron multiplier voltage at 2000 V. Perfluorokerosine was used as the reference.

D. Nuclear magnetic resonance spectra (NMR)

A WH-180 Bruker Fourier transform spectrometer was used to take NMR spectra; CDCl_3 with small amount of CHCl_3 was the solvent. The CHCl_3 peak at 7.27 ppm was used as a reference point. After the NMR spectrum was taken, a few drops of D_2O were added to the solution and another spectrum was taken to determine the extent of D_2O -exchangeable protons present.



Melting Point Determination

The melting points of some compounds were taken with a Thomas-Hoover capillary melting apparatus (A.H. Thomas Co., Philadelphia, PA).

Radioactive Isotope Labeling Study

Radioactive 2-¹⁴C-glucose and 3-¹³C-BHB were purchased from New England Nuclear Company. In order to determine what percentage of carbon in the cyst lipids was derived from glucose, an analysis was made of cysts derived from cells grown in that labeled sugar. A. vinelandii cells were grown in 50 ml of Burk's buffer plus 1% glucose plus 3.8 μ Ci of 2-¹⁴C-glucose to an OD = 0.63. Cells were harvested and washed twice and then suspended in same amount of Burk's buffer. Total radioactivity per ml of cells was determined by collecting cells on a filter and counting. Cells were then induced to encyst with unlabeled BHB, and after 5 days they were harvested and washed. The total radioactivity remaining and the radioactivity in each lipid fraction was analyzed by extracting the lipids, separating them on analytical TLC plates and counting the radioactivity in each lipid fraction. 10 ml of counting scintillant (ACS, Amersham/Searle) was shaken with lipid fraction in a vial which then was incubated in the cold overnight prior to counting in a Packard Tricarb 3320 scintillation spectrometer.



The time course of synthesis of each compound in the cyst lipids was studied by growing cells in glucose, shifting to 3-¹⁴C-BHB to induce encystment, sampling at various intervals up to 5 days during the encystment, and extracting and analyzing lipids as above.

The fate of unique cyst lipids during germination of cysts was also studied by inducing encystment in 3-¹⁴C-BHB. The cysts were then germinated in unlabeled glucose, sampled for 12 hours after germination and extracted and analyzed as above.

Because the diazotized sulfanilamide spray reagent turns compounds of interest into red-orange or yellow dyes, various degrees of quenching occur when radioactivity is monitored by scintillation. Quenching was corrected by an internal standard method. After determining the radioactivity of sprayed samples, 0.1 ml of ACS solution containing about 1000 cpm of ¹⁴C-glucose was added to each vial. After mixing and allowing the silica gel to settle, the vial was counted again and the counting efficiency of each vial was calculated as follows:

$$\text{Counting Efficiency} = \frac{\text{CPM after addition of standard} - \text{CPM before addition of standard}}{\text{CPM of standard}}$$

Thus, the radioactivity in each vial could be obtained by:

$$\text{CPM before addition of standard} / \text{counting efficiency} = \text{Corrected CPM}$$



Fractionation of Cysts

In order to study the distribution of unique cyst lipids, ^{14}C -labeled cyst (induced with ^{14}C -BHB) were fractionated into exine, intine and central bodies (50) and the lipids in each fraction analyzed.



RESULTS AND DISCUSSION

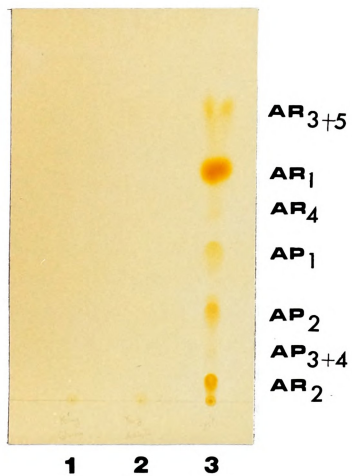
Comparison of Lipids From Vegetative Cells and Cysts

The chromatogram of lipids extracted from young vegetative cells growing exponentially in glucose or acetate, and from 5 day old cysts is shown in Fig. 1. Under the chromatographic conditions employed, all phospholipids stayed at the origin. After the TLC had developed, it was sprayed with diazotized sulphanilamide. Lipids from young vegetative cells grown in glucose or acetate do not react with the spray reagent but lipid extracts from mature cysts contain at least 9 components which react with the spray reagent. Two major components, AR_1 and AR_2 , have already been identified as mixtures of 5-n-heneicosylresorcinol and 5-n-tricosylresorcinol (AR_1) and their galactoside derivatives (AR_2) respectively (75). The other components reacting with diazosulphanilamide were designated AR_3 , AR_5 , AR_4 , AP_1 , AP_2 , AP_3 and AP_4 respectively in order of increasing mobilities. The biosynthesis of these unique lipids is correlated with the appearance of cysts or with β -hydroxybutyrate metabolism in cultures of this organism. In the solvent system I, Rf values were $AR_1 = 0.2$, $AR_5 = 0.48$ and $AR_3 = 0.75$. Other components remained very close



Fig. 1. Chromatogram of lipid extracted from A. vinelandii.

Chromatogram of lipid extracted from (1) young vegetative cells of A. vinelandii growing in 1% glucose, (2) young vegetative cells growing in 1% acetate, and (3) 5 day old cysts. Lipid extracted from 20 mg of wet cells of each type was spotted on the silica gel plate. The chromatogram was then developed in solvent system 2 (chloroform: acetone:methanol 12:4:1 v/v/v) for about 100 minutes at room temperature. After drying the plate was sprayed with diazotized sulphanilamide reagent. The 2 young vegetative cells do not have diazoreagent-reacting compounds in their lipid. But in cysts, there are at least 9 compounds in the lipid fraction reacting with diazoreagent. These were designated as AR₁, AR₂, AR₃, AR₄, AR₅, AP₁, AP₂, AP₃ and AP₄. This solvent system does not resolve AR₃ and AR₅ well. On this particular plate, AP₃ and AP₄ were not well separated.





to the origin. In solvent system II, the Rf values were $AR_1 = 0.66$, $AR_2 = 0.07$, $AR_3 = 0.78$, $AR_4 = 0.56$, $AR_5 = 0.74$, $AP_1 = 0.31$ and $AP_2 = 0.165$.

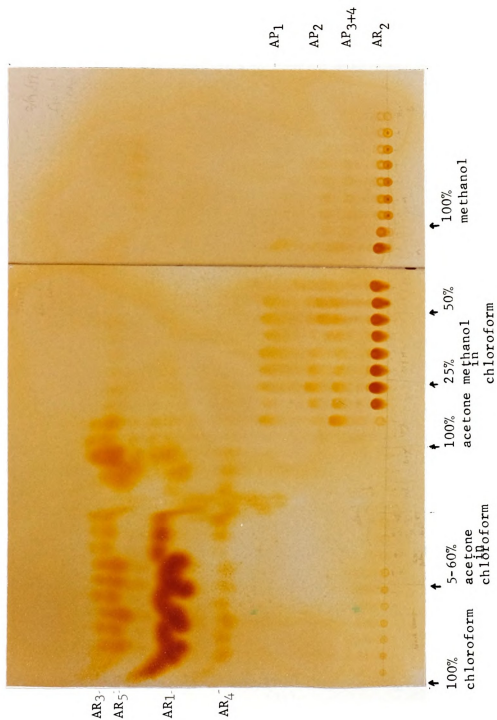
Purification of AR_3 , AR_5 , AR_4 , AP_1 and AP_2

In order to determine their chemical structures, AR_3 , AR_5 , AR_4 , AP_1 and AP_2 were purified from the mixture of lipids in the extract. AP_3 and AP_4 , which have the same color reaction as AP_2 with different spray reagents on TLC plates, were not purified because they are minor components and probably have structures similar to AP_2 . Primary separation was carried out on florisil columns. The results are shown in Fig. 2 which is a TLC analysis of the unique lipid content of the various eluates. AR_1 , AR_3 , AR_4 and AR_5 were eluted by chloroform and a chloroform acetone mixture. One hundred percent acetone starts to elute AR_2 , AP_1 , AP_4 but elution was completed with a 50% mixture of methanol in chloroform. After this treatment, most of the lipids in column had been eluted.

Fractions containing AR_1 , AR_3 , AR_4 and AR_5 were pooled and rechromatographed on a silicic acid column (cc-7). AR_3 and AR_5 were eluted together by 5% acetone in chloroform, AR_1 followed by elution with 5%-10% acetone in chloroform and AR_4 was usually eluted by 20% acetone in chloroform. Tubes from florisil columns containing AP_1 , AP_2 , AP_3 , AP_4 plus AR_2 were pooled and rechromatographed on silic acid column (cc-7). A 5-10% acetone in chloroform

Fig. 2.--Separation of A. vinelandii cyst lipid on a florisil column.

The lipid was loaded on the column and then eluted with solvents of increasing polarity as indicated with arrows. Eluate was collected in about 10 ml fractions. After the solvent evaporated, the solute in each fraction was redissolved in chloroform:methanol mixture (2:1) and analyzed with TLC (solvent system 2). Chloroform and acetone chloroform mixture elute AR₃, AR₅, AR₁, AR₄ while chloroform methanol mixture elute AP₁, AP₂, AP₃, AP₄ and AR₂ out.





mixture was used to elute AP_1 but this solvent also tended to remove AP_2 . Thus the separation of these 2 compounds was not very sharp on this kind of column. AP_3 and AP_4 were eluted after AP_2 . AR_2 was eluted by 50% acetone in chloroform and other more polar solvents. Tubes containing lipids rich in AR_3 , AR_5 , AR_4 , AP_1 and AP_2 were selected and the final purification of each component was carried out on preparative TLC. Solvent system I was used to purify AR_3 and AR_5 by TLC. Without pre-equilibration, AR_3 , AR_5 and AR_1 have R_f values of 0.75, 0.48 and 0.2 respectively in this solvent which permitted a clear separation of these 3 compounds. AR_1 usually was the major contaminant in the sample of AR_3 plus AR_5 . TLC with solvent system II and preequilibration was used to separate AP_1 , AP_2 and AR_4 . These compounds have R_f values of 0.31, 0.17 and 0.56 respectively under these conditions.

Identification of AP_1

AP_1 is a white to slightly yellow crystal which is relatively stable in air. It is soluble in hot chloroform or in a chloroform-methanol mixture but its solubility is low in cold solvent.

A direct probe mass spectrum of AP_1 is shown in Fig. 3. Upon ionization, the compound yielded a base peak at m/e 69. Other major peaks are m/e 139, 126, 111 and 98. Also of importance is the series of peaks 14 molecular weight units apart indicating a long chain hydrocarbon

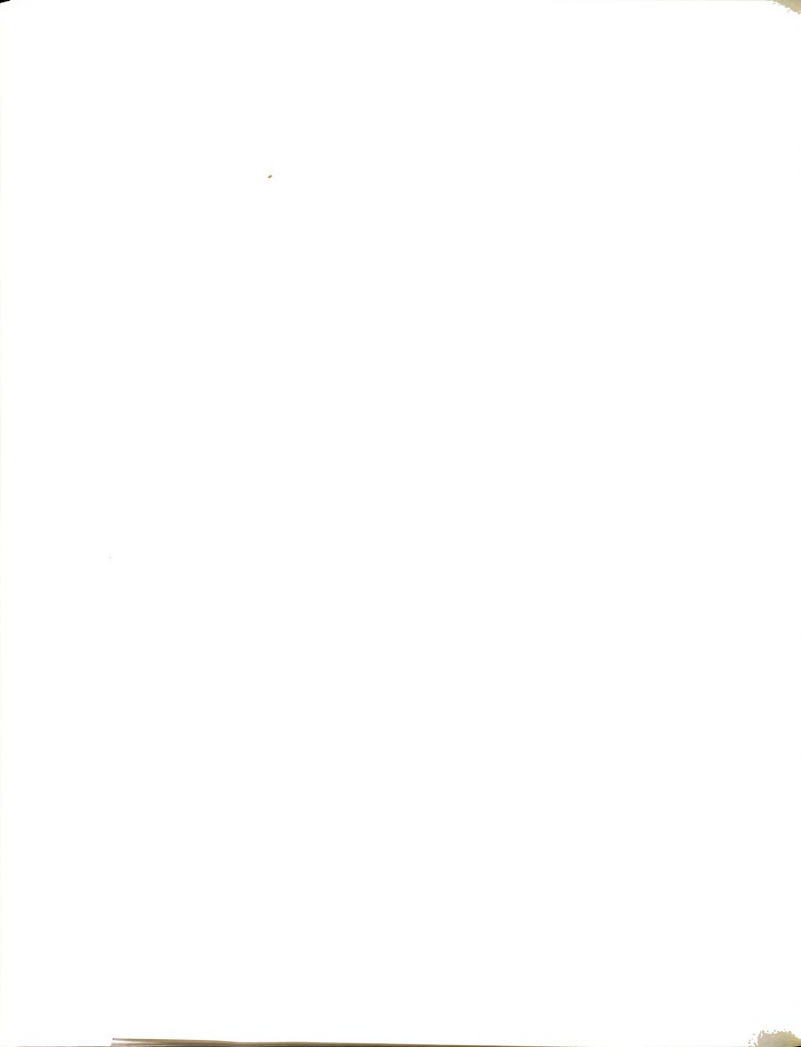
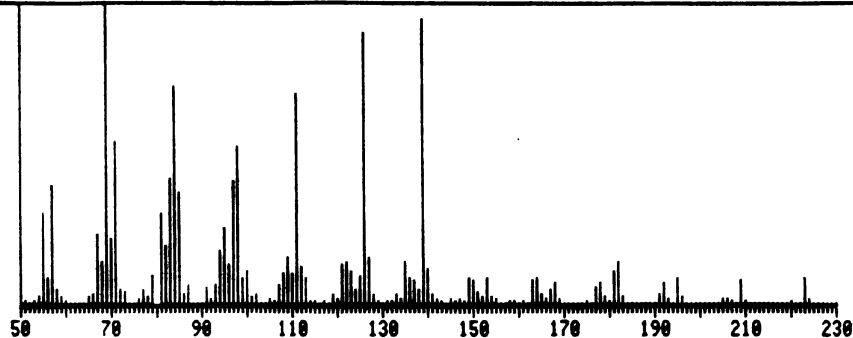


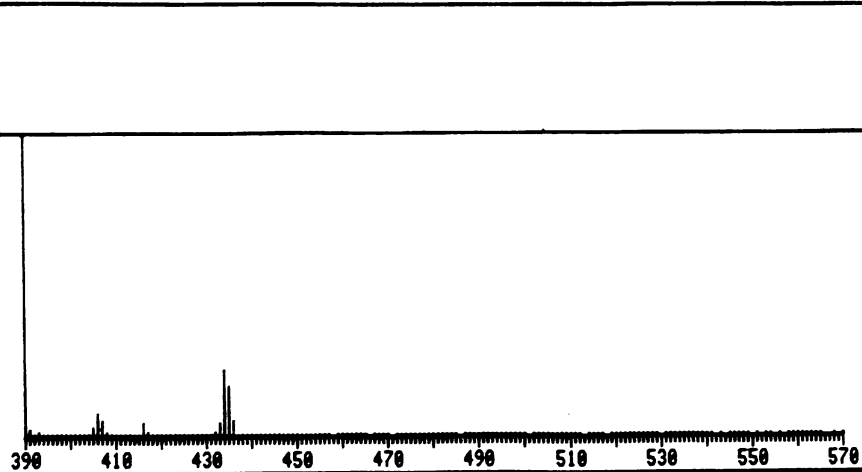
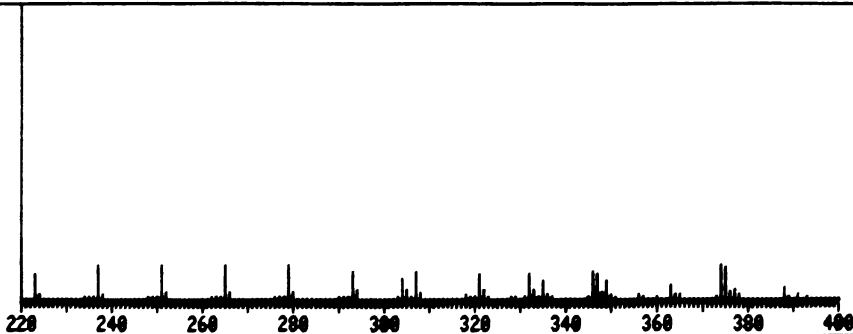
Fig. 3.--Mass spectrum of AP₁ (mixture of 2 homologues).

This spectrum was taken at M.S.U. NIH mass spectrum facility. Direct probe was the mode of sample insertion. Ionization voltage was 70 eV, and the electron multiplier was 2000 V. M/e 69 was the base peak. Other major peaks are at m/e 84, 98, 111, 126 and 139. Two molecular ions are at m/e 406 and m/e 434 respectively. A series of well spaced peaks with 14 molecular weight units apart can be observed between mass unit 180 to 300 indicating a long chain alkyl structure in the molecule.

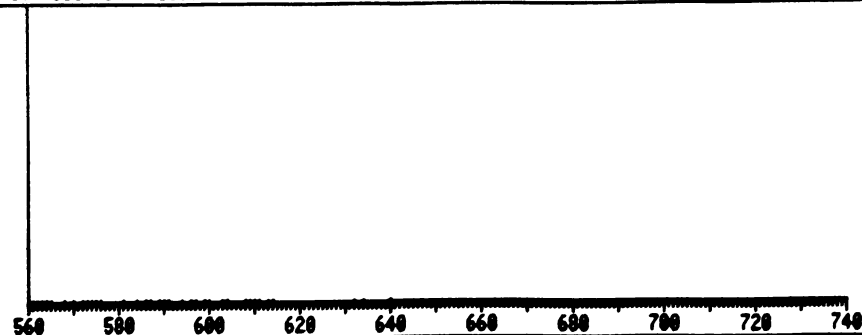
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WT 100 BRK 1000 MUL 5.00 SCH# 33 SCH# 0 %TI 0.00



AA515 SADOFF 1/29/79
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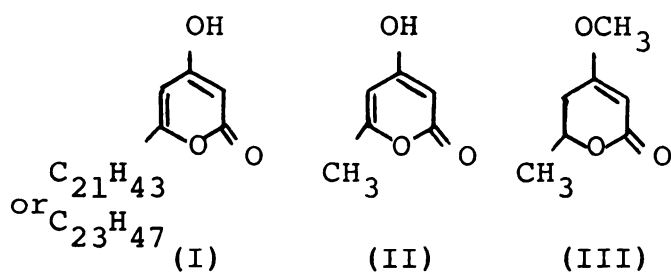
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structure. Two molecular ions were observed, m/e 406 and 434, with an intensity ratio of about 1:3.

Because I suspected that sample AP_1 was a mixture of 2 homologues, AP_1 which had been purified from preparative TLC was put through a reverse phase HPLC column (c-8). A separation of this fraction into two major components was achieved, the results of which are shown in Fig. 4. Two major peaks which had a ratio roughly 1:2.5. These were each collected and reextracted and when dried, they appeared as silver-white flakes. Mass spectral data from samples of peak I and II are similar to AP_1 with molecular ions at m/e 406 and m/e 434, respectively. The melting points of samples from peaks I and II were 107-108°C and 111-112°C respectively. Peak matching on the m/e 126, 139 and 434 peaks yielded empirical formulas for each respective peak of $C_6H_6O_3$, $C_7H_7O_3$ and $C_{28}H_{50}O_3$. Based on this information, the structure of AP_1 was proposed as a mixture of 6-n-heneicosyl-4-hydroxy-pyran-2-one and 6-n-tricosyl-4-hydroxy-pyran-2-one (I).

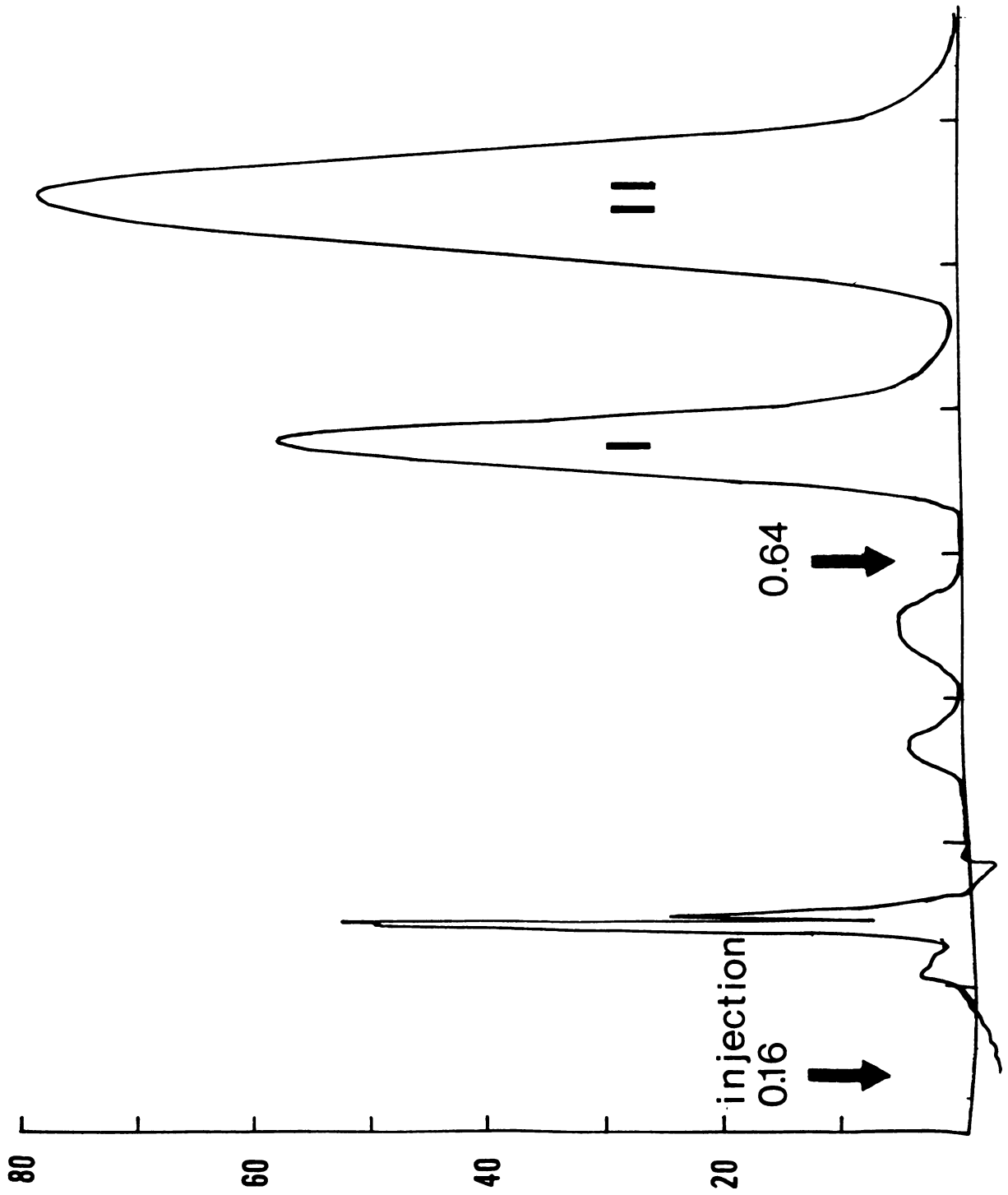


Peak m/e 126 arises from the loss of either alkene ($C_{20}H_{40}$ or $C_{22}H_{44}$) and results in the formation of 6-methyl-4-hydroxy-pyran-2-one (II). The mass spectrum of this



Fig. 4. High pressure liquid chromatography separation profile of two AP₁ homologues.

A preparative reverse phase column (Lichrosorb RP-8) was used. The mobile phase was 10% water in methanol. The solvent was pumped at 2 ml/minute at a pressure of 1200-600 pound per square inch. The chart speed was at 8 inches per hour and the detector wavelength was set at 280 nm. The detector sensitivity was set at 0.16 optical density was full scale at the beginning of the run and decreased to 0.64 at the second arrow. The first sharp peak was probably due to the solvent used to dissolve the sample. Two major peaks, I and II, were identified as 6-heneicosyl-4-hydroxy-pyran-2-one and 6-tricosyl-4-hydroxy-pyran-2-one respectively. These 2 peaks have a ratio of about 1:2.5. The whole process required about 1 hour and only 2 small contaminating peaks were observed in AP₁ sample purified on preparative TLC.

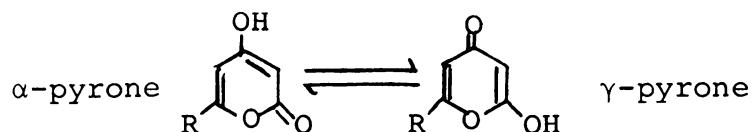




compound has 5 major peaks at m/e 69, 43, 98, 126 and 85 (91). The compounds I have isolated also produce these peaks and therefore match very well with this structure.

The methylation of AP₁ results in the formation of a yellowish crystal. Gas chromatography separates this mixture into 4 peaks. GC-MS analysis of each peak yielded molecular ions of m/e 420, 420, 448 and 448 indicating that the addition of 1 methyl group on each of homologues had occurred. This confirmed my proposed structure of only one -OH group. Peaks of m/e 43, 41, 125, 153, 69 and 140 were obtained which also corresponded to the mass spectrum of 6-methyl-4-methoxy-pyran-2-one (III) which has major peaks at m/e 112, 69, 125, 43 and 140 (91).

The 4 derivatives of methylation can be explained on the basis that when diazomethane was used to methylate pyrone, it yielded a mixture of α and γ methylated pyrones (34) because pyrone exists as a mixture of 2 tautomers under acidic conditions.



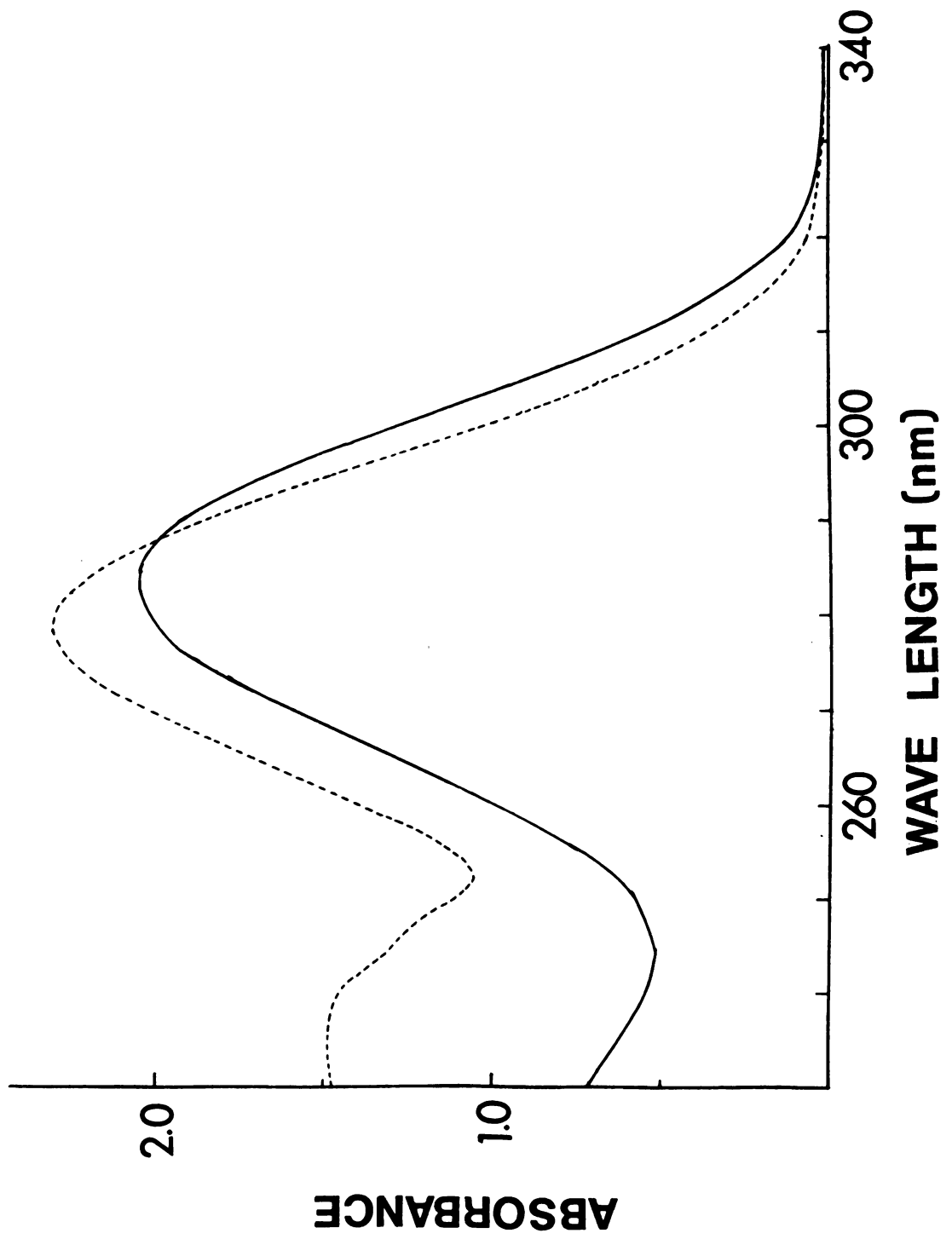
Thus with 2 homologues, methylation produced 4 derivatives which was confirmed by GC-MS analysis.

The UV absorption spectrum of AP₁ in 95% ethanol is shown in Fig. 5. It had an absorption maximum at 283 nm and a minimum at 241 nm. Using a molecular weight of 434,



Fig. 5. UV absorption spectrum of AP_1 .

The solvents were 95% ethanol (—) and 0.67% KOH in ethanol (-----).
Sample concentration was 1.203×10^{-4} M.





the calculated extinction coefficient was $\epsilon = \log 4.09$ at 283 nm. It also had an abnormal hypsochromic shift in alkaline solution $\lambda_{\max}^{\text{EtOH-KOH}}$ 278 nm $\epsilon = \log 4.32$. This phenomenon has been observed in the 4-hydroxy-2-pyrones and their enol tautomers (7, 8). The vanillin test on AP_1 was negative. Both observations exclude the possibility that AP_1 is a phenolic compound (75). The high extinction coefficient indicated that AP_1 was a ring structure, but the value was too high for an aromatic compound. The absorption wavelength indicated that AP_1 was a conjugated chromophore. The UV spectrum is similar to the compounds triacetic lactone (6-methylpyranone) ($\lambda_{\max}^{\text{H}_2\text{O}}$ 288 nm $\epsilon = 6750$) (61, 99) and 3,6-dimethyl-4-hydroxy-2-pyrone ($\lambda_{\max}^{\text{EtOH}}$ 288 nm $\epsilon = 8300$) (1) which are similar to our proposed structure. Because γ -pyrones have absorption peaks around 240 nm, my spectral results suggests that AP_1 exists principally as the α -pyrone.

The infra red (IR) absorption spectrum is shown in Fig. 6. AP_1 has absorption peaks at 2940 and 2880 cm^{-1} (aliphatic C-H), 1680 cm^{-1} (C=O), 1645 and 1565 cm^{-1} (C=C), 1300 cm^{-1} (C-O stretching), 1250 cm^{-1} (Ar-O vibration) and 1145 cm^{-1} (C-O stretching). After methylation, IR absorption was observed at 2950 and 2880 cm^{-1} (aliphatic C-H), 1725 cm^{-1} (C=O), 1570 cm^{-1} (C=C) and 1140 cm^{-1} (C-O stretching).

The nuclear magnetic resonance (NMR) spectrum of AP_1 in CDCl_3 is shown in Fig. 7. Using the CHCl_3 peak as a reference at 7.27 ppm, 5 absorption peaks were observed at 5.9 (singlet), 5.5 (singlet), 2.48 (triplet), 1.29 (multiple),

Fig. 6. Infrared absorption spectrum of AP_1 .
The spectrum was taken as Nujol mull.

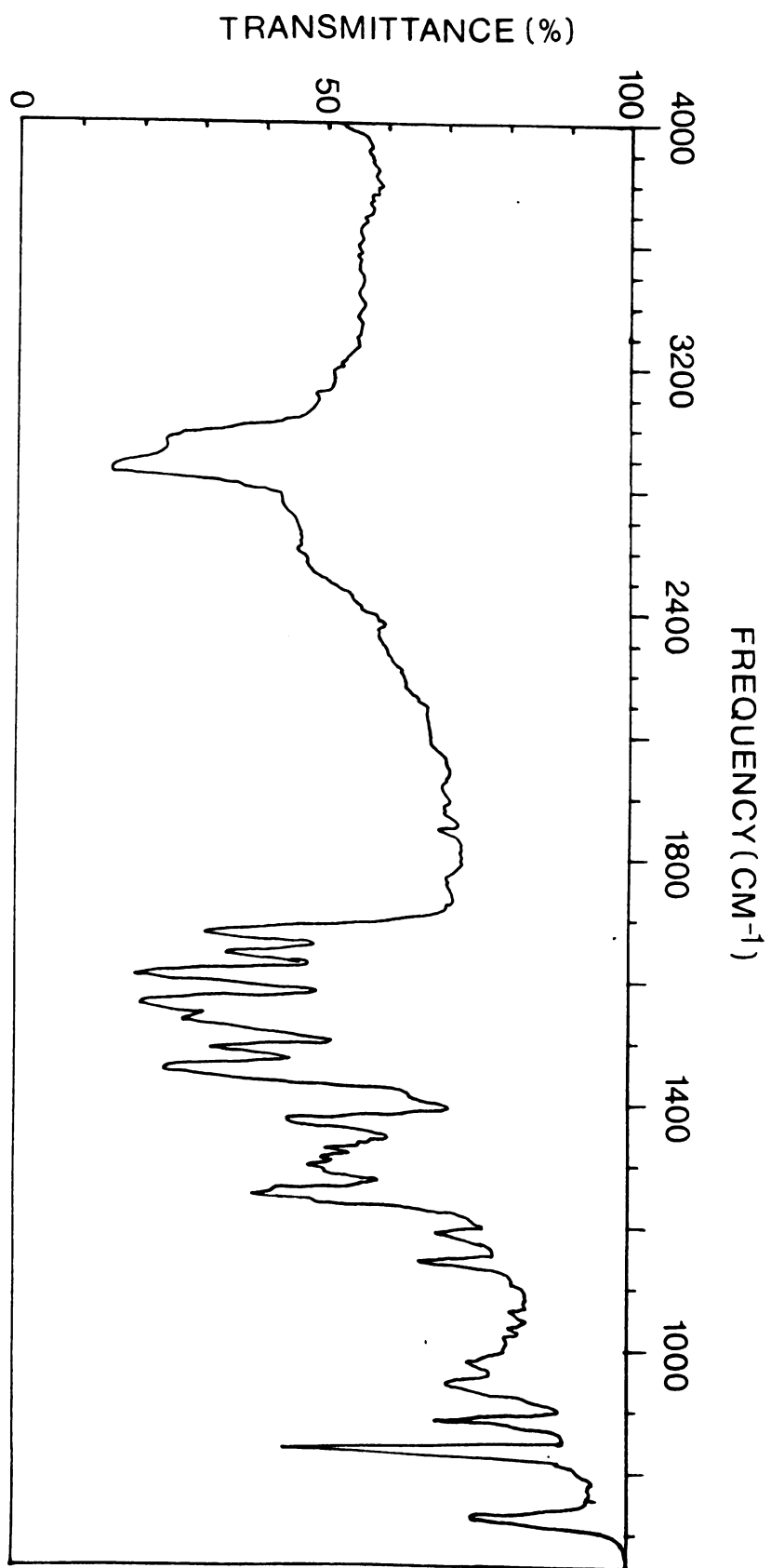
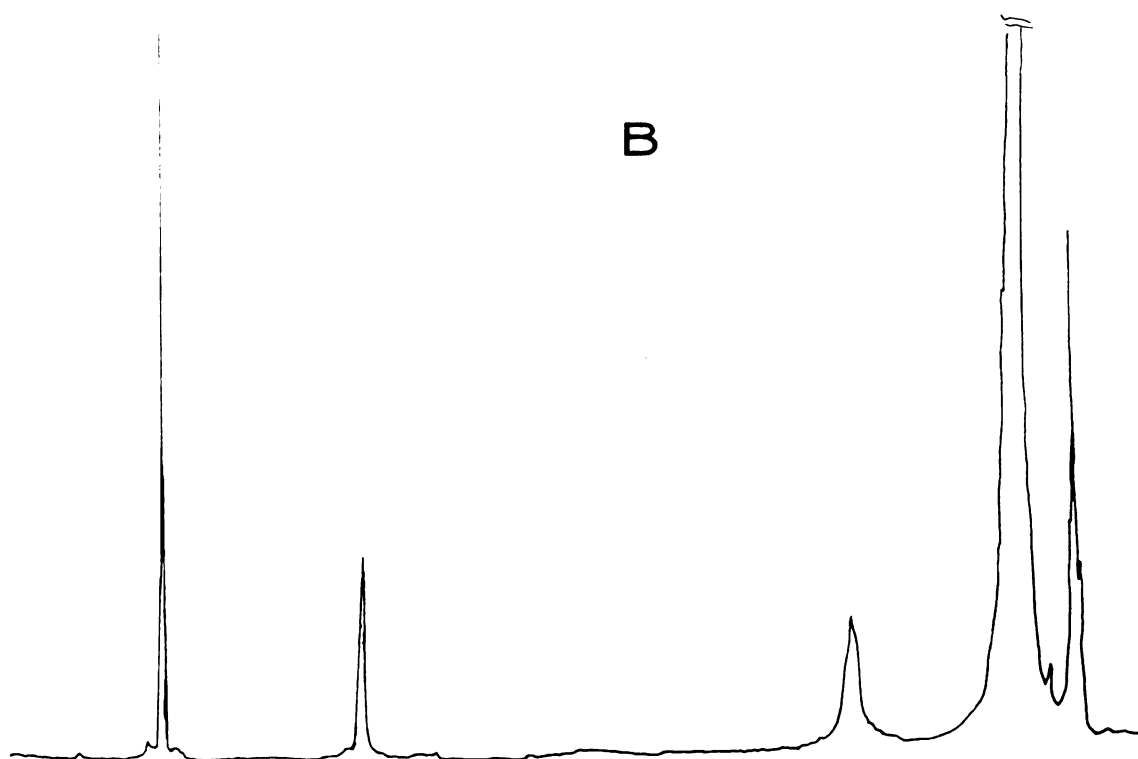
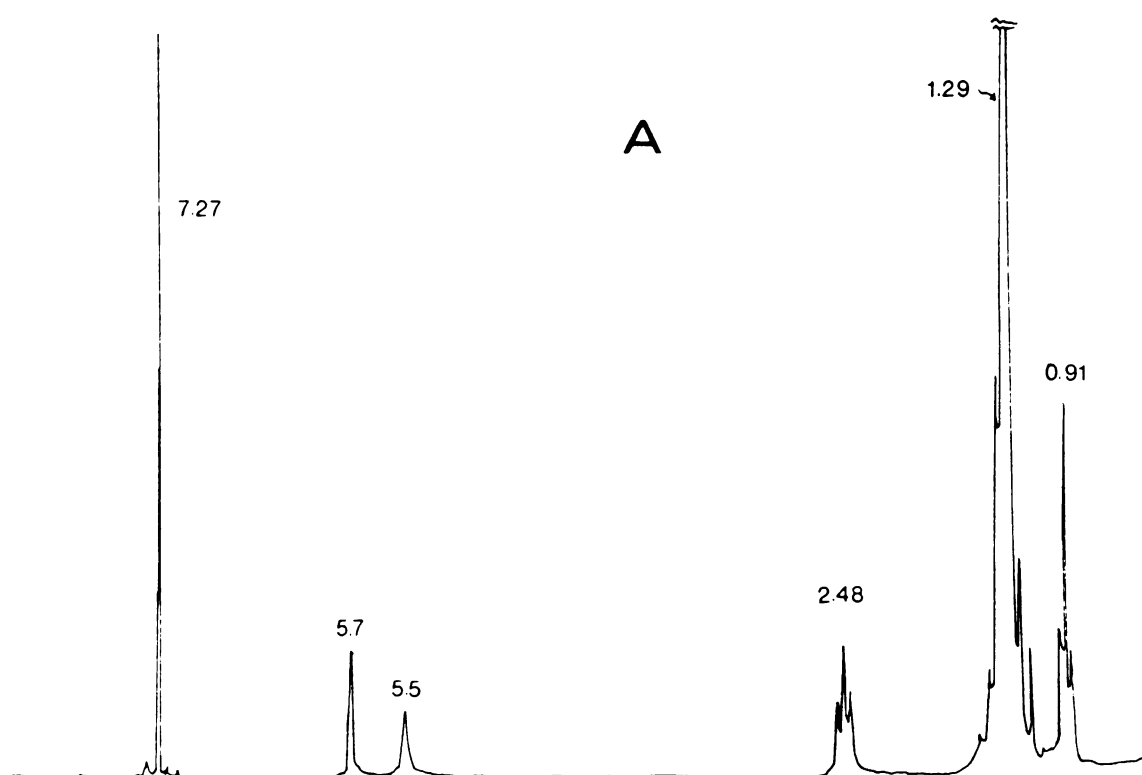




Fig. 7. NMR spectrum of AP₁.

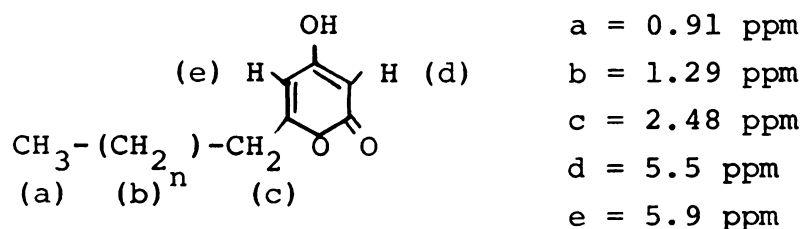
CDCl₃ was used as solvent. The sharp peak at 7.27 ppm was the CHCl₃ peak.

- A. Before the addition of D₂O.
- B. After addition of D₂O to the AP₁ solution, the small peak at 5.5 ppm disappeared.

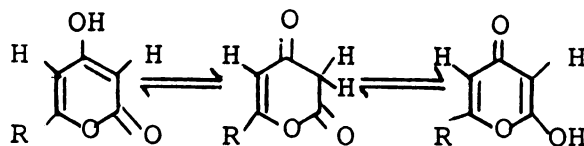




and 0.91 (triplet) ppm respectively with a relative intensity of 1:1:2:40:3. These protons are assigned as follows:



The proton on the -OH group was not detected. The assignment of d and e protons are according to the literature (16). Upon the addition of D₂O into the AP₁ solution, the peak at 5.5 ppm disappeared. Its disappearance was explained by the fact that proton d is an exchangeable proton because of the resonance between 2 tautomers.



The NMR spectrum obtained was comparable to standard NMR data from 4-hydroxy-6-methyl-2H-pyran-2-one which has peaks at 6.25 (e), 6.21 (d) and 2.47 (-CH₃) ppm respectively.

Identification of AP₂

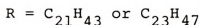
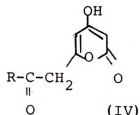
AP₂ is a silver-white to slightly yellow crystal with a distinct smell. It is unstable and turns yellow to brownish after exposure to air. It is also unstable in either acid or alkaline conditions. AP₂ is soluble in a chloroform-methanol mixture and its melting point is 97-101°C.

AP₂ was separated into 2 components of roughly the same concentration on a reverse phase HPLC column in which 20% H₂O in methanol was used as the mobile phase. The direct probe mass spectrum of the first component eluted yielded a base peak at m/e 153. Other important mass spectral peaks were m/e 168, 181, 43 and 430. The m/e 430 peak was thought to be the molecular ion. The results of peak matching yielded the following empirical formulas m/e 153 (C₇H₅O₄), 168 (C₈H₈O₄), 181 (C₉H₉O₄) and 430 (C₂₈H₄₆O₃). Because the m/e 430 peak had one oxygen less than the other peaks, I suspected that a loss of H₂O occurred during mass spectrometry and that a smaller peak at m/e 448 was the true molecular ion (C₂₈H₄₈O₄). A sample of the second compound eluted from HPLC give a similar mass spectrum with a molecular ion at m/e 476.8. Peak matching on peak m/e 153, 168 and 181 yielded the same empirical formula as obtained for the first homologue of AP₂ eluted from HPLC. M/e 458 peak gave a formula of C₃₀H₅₀O₃, which, once again, suggested an initial loss of one water molecule. I concluded that peak 2 of AP₂ was the homologue of Peak I with 2 more carbon atoms in the alkyl chain and with an empirical formula of C₃₀H₅₂O₄.

Because electron impact ionization was suspected to produce an initial loss of water from the homologues in AP₂, chemical ionization was used in mass spectral analysis of AP₂. The spectrum had a base peak at m/e 127 and the 2

molecular ions at m/e 448 and 476 were confirmed. There was no apparent loss of water from the molecular ions in this spectrum.

With additional information from UV, IR and NMR, the possible structure of AP_2 was postulated as 6-(2-oxo-tricosyl)-4-hydroxy-pyran-2-one and 6-(2-oxo-pentacosyl)-4-hydroxy-pyran-2-one (IV), which fits most of the data,



Methylation of AP_2 resulted in the formation of a yellowish crystal. GC-MS analysis revealed a mixture of more than 10 compounds and most of these had molecular weights less than the mother compound. I concluded that the methylating reagent and treatment (diazomethane) caused decomposition of AP_2 .

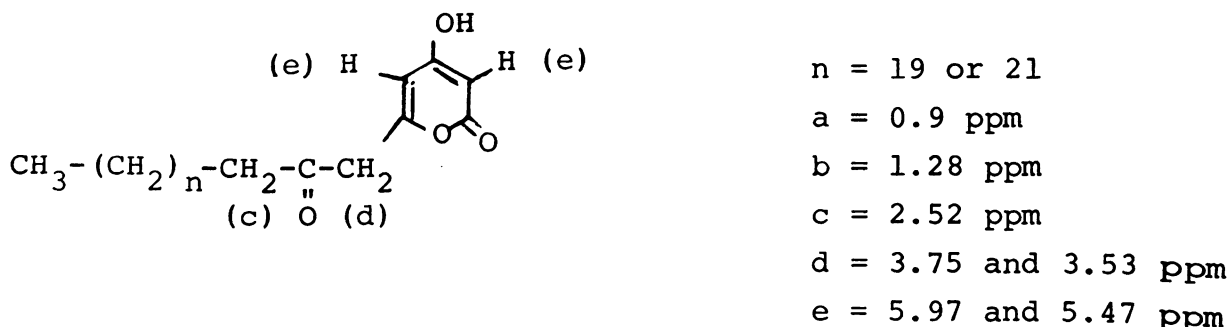
The UV absorption of AP_2 was similar to the AP_1 absorption spectrum with $\lambda_{\max}^{\text{EtOH}}$ 286 nm and $\log \epsilon = 3.72$. The extinction coefficient was calculated using an average molecular weight of 462 for AP_2 . The minimum absorption occurred at 250 nm. Upon the addition of KOH to a solution of AP_2 , 2 absorption maximum were observed at $\lambda_{\max}^{\text{EtOH}}$ 260 nm, $\log \epsilon = 3.97$ and $\lambda_{\max}^{\text{EtOH}}$ 360 nm, $\log \epsilon = 4.176$, respectively, with an increase in extinction coefficient. These results



agree very well with the UV absorption data obtained from 6-(2-oxopropyl)-4-hydroxy-2-pyrone (6).

The IR spectrum of AP₂ has peaks at 3475 cm⁻¹ (-OH), 2920 and 2850 cm⁻¹ (aliphatic C-H), 1675 cm⁻¹ (C=O stretching), 1260 cm⁻¹ (Ar-O vibration), 720 cm⁻¹ (CH₂ rocking) and 1140 cm⁻¹ (C-O-C).

The NMR spectrum has 7 peaks at 5.97 (singlet), 5.47 (singlet), 3.75 (singlet), 3.53 (singlet), 2.52 (triplet), 1.28 (multiplet) and 0.9 (triplet) ppm each with a ratio of peaks of about 0.5:0.2:1:1:2:40:3. The assignment of each peak is as follows:



The reason that proton d exists as 2 peaks is probably the tautomerization of the α - and γ -pyrones.

All the data from AP₂ fit very well with the description of the data from 6-(2-oxopropyl)-4-hydroxy-2-pyrone (6) except for the loss of H₂O in the electron impact mass spectrum. This difference could be due to the fact that AP₂ has a longer side chain or alternatively, due to different conditions used to take the mass spectrum (e.g.: Electron voltage used to ionize the molecule).



Identification of AR₃

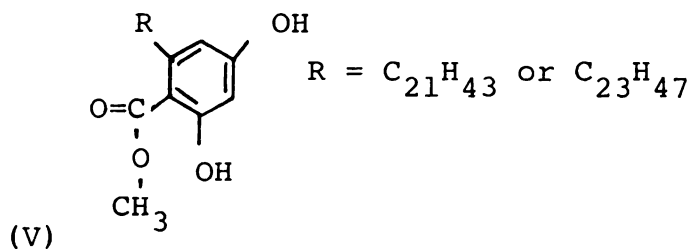
AR₃ which had been purified from preparative TLC was a white to pale yellow crystal melting at 95-97°C. It was unstable in air and soluble in a mixture of chloroform and methanol. On silica gel plates, it turned red to purple on standing in the air or after exposure to iodine vapor. Its vanillin test for phenol was positive and it reacted with the diazoreagent to form a red color. The AR₃ reaction to these test reagents was similar to that of AR₁ (75).

Direct probe mass spectral analysis of AR₃ produced a base peak at m/e 182. Other important peaks were m/e 150, 163, 124, 123, 43 and 57, molecular ions were at m/e 462 and 490. There were many peaks with 14 molecular weight differences in the range 200 to 400 molecular weight indicating that the compound possessed a long chain hydrocarbon. The results of peak matching on several peaks were as follows: m/e 150 (C₈H₆O₃), 163 (C₉H₇O₃), 163 (C₁₀H₁₁O₂) and 182 (C₉H₁₀O₄). If 182 were subtracted from 462 and 490, the respective remainders would be 280 and 308 which are exactly the molecular weights of C₂₀H₄₀ and C₂₂H₄₄ respectively. From the above information, I concluded that AR₃ homologues are derivatives of AR₁ (5-n-heneicosylresorcinol and 5-n-tricosylresorcinol) with substituent C₂H₂O₂ on the aromatic ring.

Methylation of AR₃ resulted in a yellowish crystal. Mass spectral analysis of this material yielded a base peak at m/e 196. Other major peaks were m/e 164, 177 and 210.



The m/e 196 and 210 peaks probably were derived from peak m/e 182 of the mother compound with the addition of 1 or 2 methyl groups. Molecular ions were at m/e 476, 490, 504 and 518 and represent the addition of either one or two methyl groups to the original 2 homologues. The mass spectrum also shows a long chain hydrocarbon in the molecule. I conclude that there are two exchangeable protons in the molecule and that these probably are the 2 hydroxy groups on the aromatic ring. Based on all information available, the proposed structure of the two AR₃ homologues are 6-n-heneicosylresorcylic acid methyl ester and 6-n-tricosylresorcylic acid methyl ester (V) in a ratio of about 1:1.

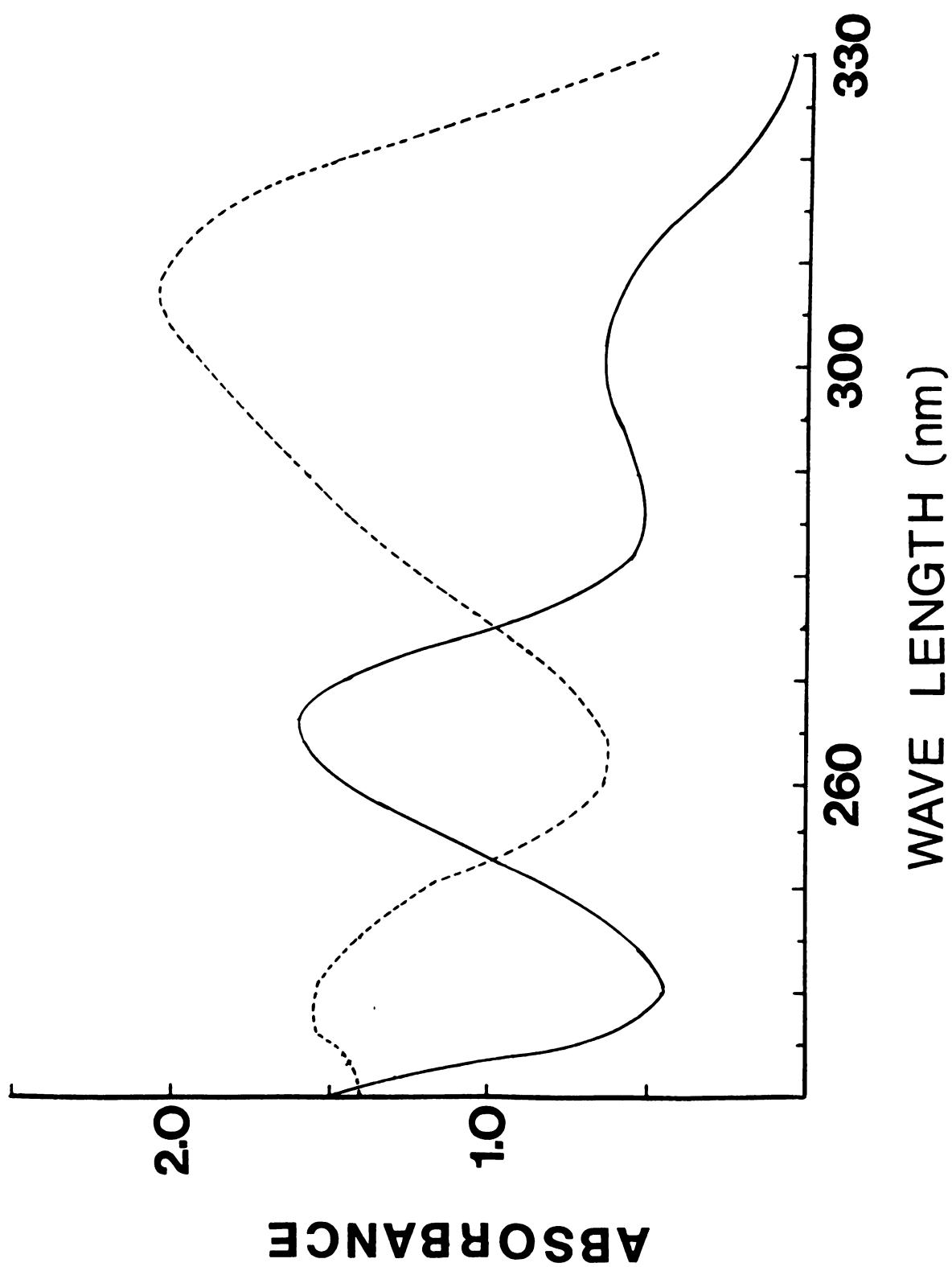


The UV absorption spectrum of AR₃ are shown in Fig. 8, it has 2 maxima at $\lambda_{\max}^{\text{EtOH}}$ 265 nm, $\log \epsilon = 3.99$ and $\lambda_{\max}^{\text{EtOH}}$ 300 nm, $\log \epsilon = 3.59$ and 2 absorption minima at $\lambda_{\min}^{\text{EtOH}}$ 240 nm, $\log \epsilon = 3.465$ and $\lambda_{\min}^{\text{EtOH}}$ 285 nm, $\log \epsilon = 3.496$. The addition of 0.1 M KOH to a solution of AR₃ results in the shift of its absorption peak to 304 nm and an increase of its extinction coefficient to $\log \epsilon = 4.1$. These results indicate that AR₃ is a phenolic compound. Mosbach



Fig. 8. UV absorption spectrum of AR_3 .

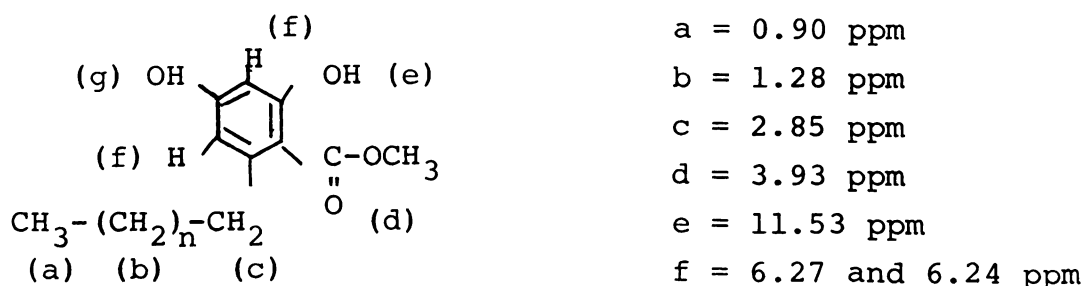
The solvents were 95% ethanol (—) and 0.1 N KOH in 95% ethanol (-----).
Sample concentration was 1.623×10^{-4} M.



(64) reported that the UV absorption spectrum of orsellinic acid had a $\lambda_{\max}$ 260 nm, $\epsilon = 9750$ and $\lambda_{\max}$ 296 nm in 0.1 N HCl. These results are similar to values for AR₃.

The IR spectrum of AR₃ in CCl₄ has absorption peaks at 3590 and 3270 cm⁻¹ (-OH), 2950 and 2875 cm⁻¹ (aliphatic C-H), 1440 cm⁻¹ (CH-C), 1720, 1650, 1320, and 1110 cm⁻¹ (C=O and ester), 1620, 1585, 950, and 850 cm⁻¹ (benzene substitution) and 1260 cm⁻¹ (aromatic -OH). These results compare well to the IR absorption of β -resorcylic acid methyl ester.

The NMR spectrum of AR₃ had 6 peaks at 11.53 (singlet), 6.27 and 6.24 (multiplet), 3.93 (singlet), 2.85 (triplet), 1.28 (multiplet) and 0.9 (triplet) ppm respectively. The intensity ratio of the peaks was approximately 1:2:3:2:40:3. The assignment of these protons was as follows:



The 2 aromatic ring protons absorb at 2 different ppm indicating an asymmetric molecule. Thus, the position of methylcarbonyl group had to be at 4 position on the ring. Proton e shifts to the high field because of hydrogen bonding between the -OH and the C=O group. The intensity



of the peak diminished when D_2O was added to the solution. The other $-OH$ was not detected in the NMR. Thus all data obtained confirm the proposed structure of AR_3 .

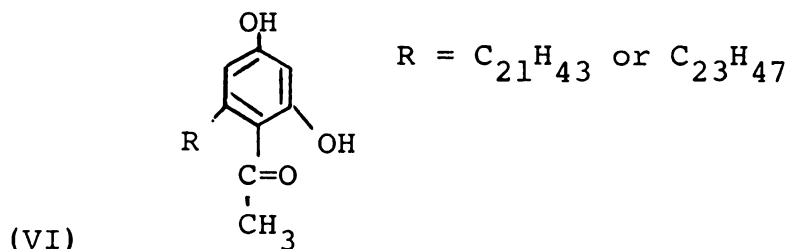
Identification of AR_5

AR_5 purified from TLC was a white to pale yellow crystal which turned yellow on exposure to air. It had a melting point of $94-95^\circ C$ and was stable in a mixture of chloroform and methanol. On silica gel plates, it turned red to purple upon exposure to air or iodine vapor. The vanillin test for phenol was positive and its reaction when sprayed with the diazoreagent yielded a red dye.

Direct probe mass spectral analysis of AR_5 yielded a base peak at m/e 124. Other major peaks were m/e 123, 137, 166 and 404. A series of peaks, 14 molecular weight units apart, also was present between 180-300 indicating the presence of a long chain hydrocarbon on the molecule. Two small peaks at m/e 446 and 474 were suspected to be the molecular ions. The mass spectrum of chemically ionized AR_5 confirmed that the molecular ions are m/e 446 and 474 in a ratio of 6:4 respectively.

Methylation of AR_5 results in the formation of a yellowish crystal. Mass spectral analysis of this material yielded a base peak at m/e 138 (1 methylation of 124 peaks). Other important peaks were m/e 152 (2 methylations), 124 and 43. Other peaks present were m/e 180 (1 methylation of 166 peak); 418 and 460 (1 methylation of 404 and 446

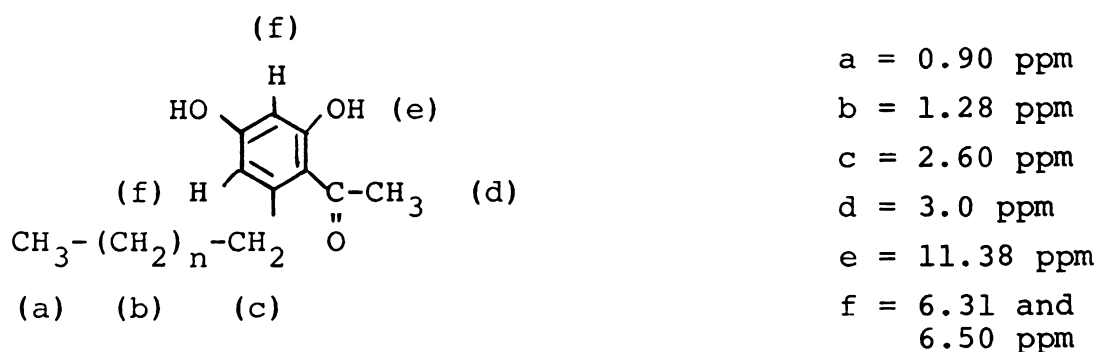
respectively) and 432 (2 methylations of 404) indicating there were 2 exchangeable protons in the molecule. This compound was thought to be very similar in structure to AR_3 and a derivative of AR_1 with an extra group of 42 molecular weight. The peak at m/e 166 was thought to be the ion resulting from the β -cleavage and loss of side chain as alkenes ($C_{20}H_{40}$ or $C_{22}H_{44}$). The data suggest that the functional group $CH_2-C(=O)$ was the substituent of mass 42 and the proposed structure of AR_5 homologues are 5-n-heneicosyl-4-acetyl-resorcinol and 5-n-tricosyl-4-acetyl-resorcinol (VI)



The UV absorption spectrum of AR_5 has 2 absorption peaks at λ_{max}^{EtOH} 271 nm, $\log \epsilon = 3.935$ and λ_{max}^{EtOH} 305 nm, $\log \epsilon = 3.57$. Two absorption minima are at λ_{min}^{EtOH} 242.5 nm and λ_{min}^{EtOH} 295 nm. A bathochromic shift and increase of extinction coefficient was observed upon the addition of KOH to the solution of AR_5 indicating the presence of a phenolic group. The UV absorption spectrum and extinction coefficient of AR_5 was very similar to AR_3 indicating a similar chromophore.

The IR spectrum of AR₅ in CCl₄ has absorption peaks at 3620 cm⁻¹ (-OH), 2960 and 2870 cm⁻¹ (C-H), 1720 cm⁻¹ (C=O), 1605 and 1585 cm⁻¹ (aromatic ring substitution), 1455 cm⁻¹ (C-H) and 1215 cm⁻¹ (phenolic group).

The NMR spectrum of AR₅ has six peaks at 11.38 (singlet), 6.5 (singlet), 6.31 (singlet), 3.0 (triplet), 2.60 (triplet), 1.28 (multiplet) and 0.9 (triplet) ppm respectively. The intensity ratio of these peaks is about 1:1:1:3:2:40:3. The assignment of these peaks are as follows:



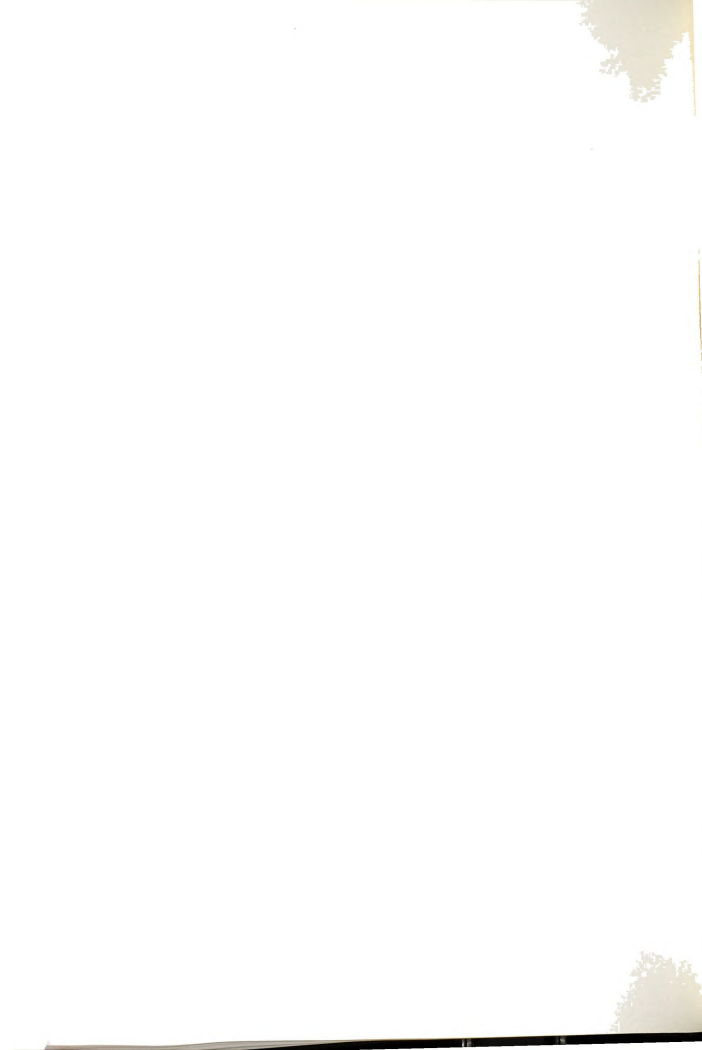
Two different absorption peaks of protons on the benzene ring indicate an asymmetric molecule and thus the location of the acetyl group has to be at carbon 4 of the ring. The proton absorption at high field indicated hydrogen bonding between an -OH and a group (C=O) ortho to it.

Identification of AR₄

AR₄ which had been purified by preparative TLC was a white crystalline compound of melting point 95-101°C. It was unstable and turned brown upon exposure to air. It was insoluble in a chloroform but soluble in a

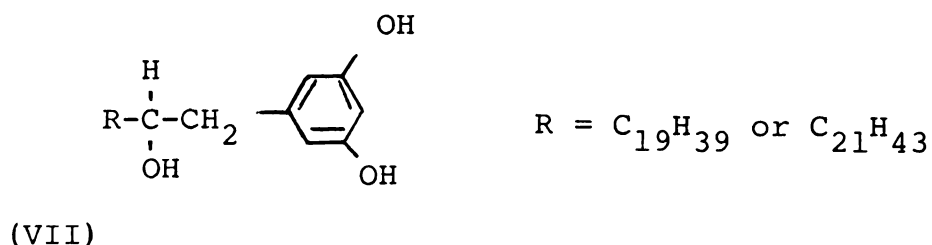
chloroform-methanol mixture. On silica gel plates, it turned red or purple upon exposure to air or iodine vapor. Its vanillin test for phenol was positive and it reacted with diazosulfanilamide to produce a red dye.

The direct probe mass spectrum of AR₄ had a base peak at m/e 124. Other important peaks were m/e 123, and 137, results which were very similar to the AR₁ spectrum (75). Two molecular ions were noted at m/e 420 and m/e 448 respectively. Peak matching on peak 448 yielded an empirical formula of C₂₉H₅₂O₃. AR₄ consumed large amounts of diazomethane upon methylation. After methylation, it became a white to yellowish crystal. A short GC column (61 cm, SE-30) was used to separate the methylated AR₄ into 2 broad components (A and B) with a peak ratio of 3:4 for the 2 homologues. A longer column (183 cm SE-30) resolved each individual component (A or B) into 3 fractions for a total of 6 components in methylated AR₄. GC-MS analysis of each of the three compounds derived from fraction A revealed two or three methyl groups on each homologue and a small amount of a tetramethyl derivative. The mass data for these methylated derivatives were m/e 152 (base peak) and 488 (molecular ion), m/e 166 (base peak) and 462 (molecular ion) and m/e 180 (base peak) and 476 (molecular ion). These data indicate that the compounds separated by the GC peaks are 2, 3 and 4 methyl group-containing derivatives of the fraction A. The other 3 components (methylated derivatives of the AR₄ fraction B) were 28 molecular weight units



heavier than those derived from fraction A. The fact that derivatives containing 3 methyl groups had base peaks of 166 suggested that only 2 hydroxy groups could exist on the ring (the molecular weight of tri-methoxy toluene is 182) and that the other oxygen was probably on the side chain as an alcohol.

The following structure of AR_4 was proposed (VII)



5-n-(2-hydroxy)-heneicosylresorcinol and 5-n-(2-hydroxy)-tricosylresorcinol.

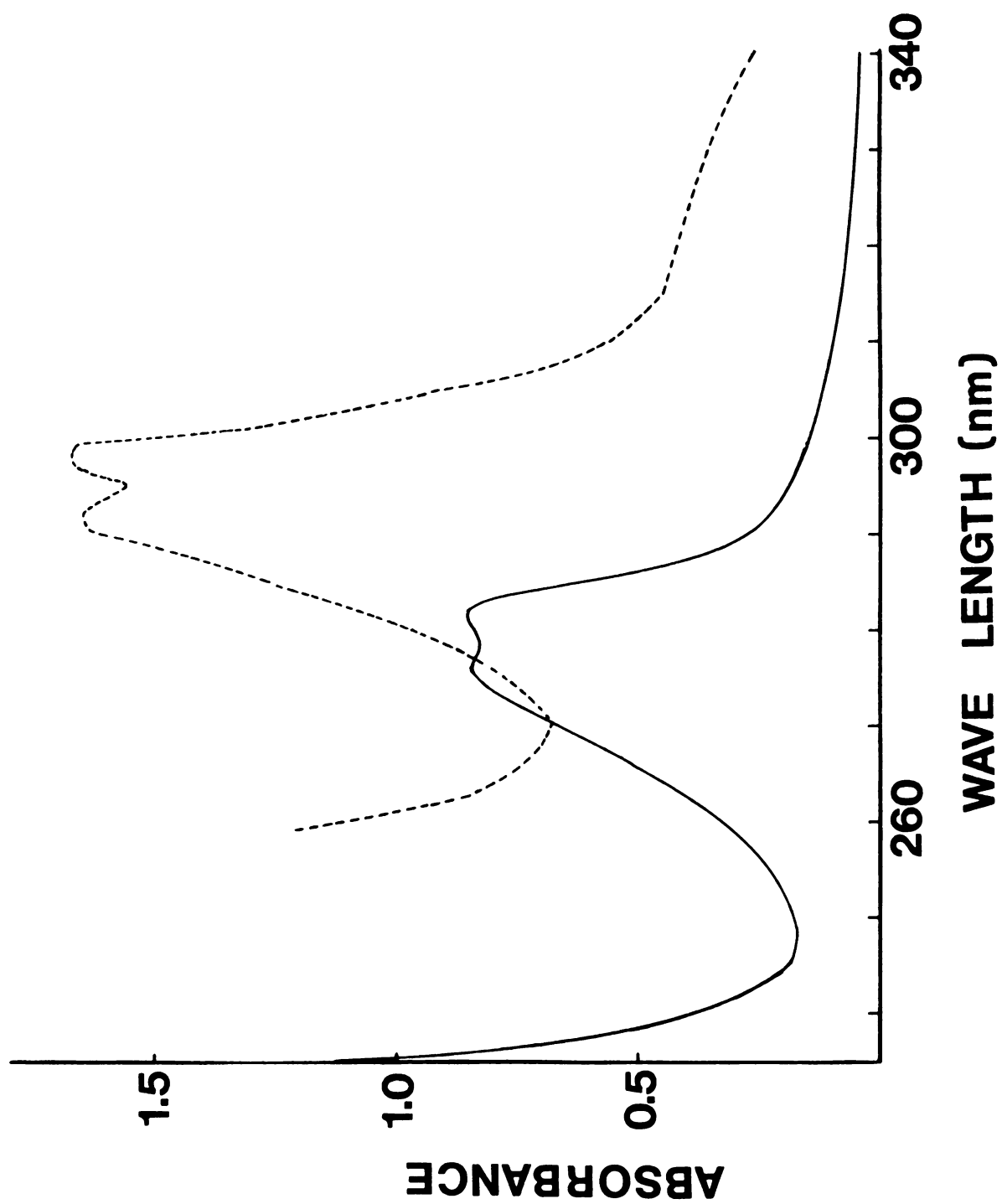
The UV absorption spectrum of AR_4 was shown in Fig. 9, it was very similar to the spectrum of AR_1 (75) having 2 absorption peaks at $\lambda_{\max}^{\text{EtOH}}$ 281 nm, $\log \epsilon = 3.199$ and $\lambda_{\max}^{\text{EtOH}}$ 275 nm, $\log \epsilon = 3.19$. Upon the addition of alkali (1N KOH) there occurred a shift in the absorption peak to 297.5 and 292.5 nm with an increase of extinction coefficient. These data indicate the presence of phenolic groups.

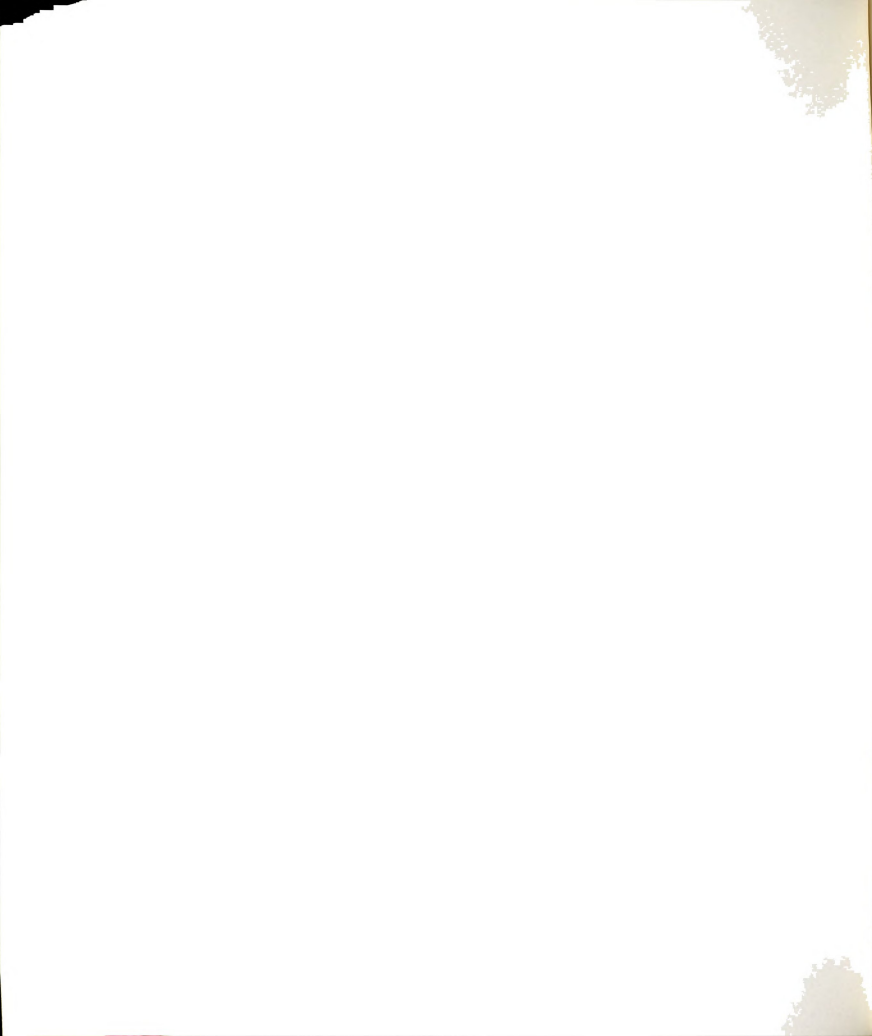
The IR spectrum of AR_4 had a very broad and strong peak at 3300 cm^{-1} (-OH group), 2890 and 2820 cm^{-1} (aliphatic C-H), 1600 and 830 cm^{-1} (benzene substitution), 1160 cm^{-1} (aromatic alcohol), and 1470 cm^{-1} (CH-C).



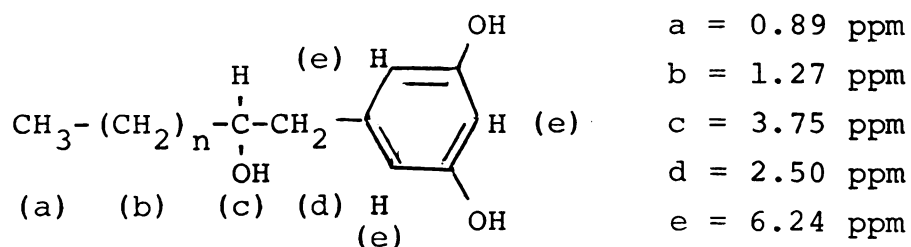
Fig. 9. UV absorption spectrum of AR_4 .

The solvents were 95% ethanol (—) and 0.1 N KOH in 95% ethanol (-----).
Sample concentration was 3.33×10^{-4} M.





The NMR spectrum of AR₄ had 5 major peak at 6.24 (multiplet), 3.75 (singlet), 2.50 (multiplet), 1.27 (multiplet) and 0.89 (triplet) ppm each. The ratio of these peaks was approximately 3:1:2:40:3. Assignment of these protons is as follows:



The structures and properties of unique lipids from encysting A. vinelandii are presented in Table 1.

The Origin of Carbon Atoms in Unique Cyst Lipids

The synthesis of unique lipids occurred after the induction of encystment at which time BHB was the only carbon source available. Therefore, it is very likely these compounds were derived from BHB. This possibility was investigated by using 3-¹⁴C-BHB to induce encystment and determining the distribution of radioactive carbon in cyst components. The total uptake of BHB, the radioactivity incorporated in the lipid fraction, as well as the change in culture turbidity were followed for 5 days and the results are shown in Fig. 10. The culture turbidity increased from OD = 0.6 to 1.0 within 24 hours after induction of encystment and reflects an increase of cell number. After reaching the population peak, the culture



Table 1.--Structures and Properties of Unique Lipids from Encysting *A. vinelandii*.

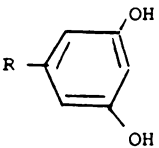
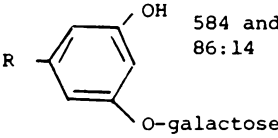
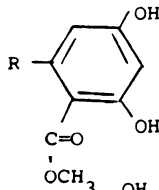
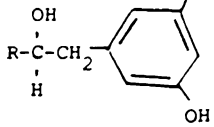
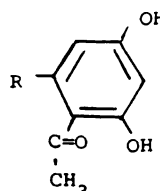
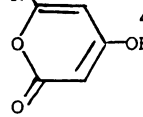
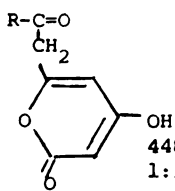
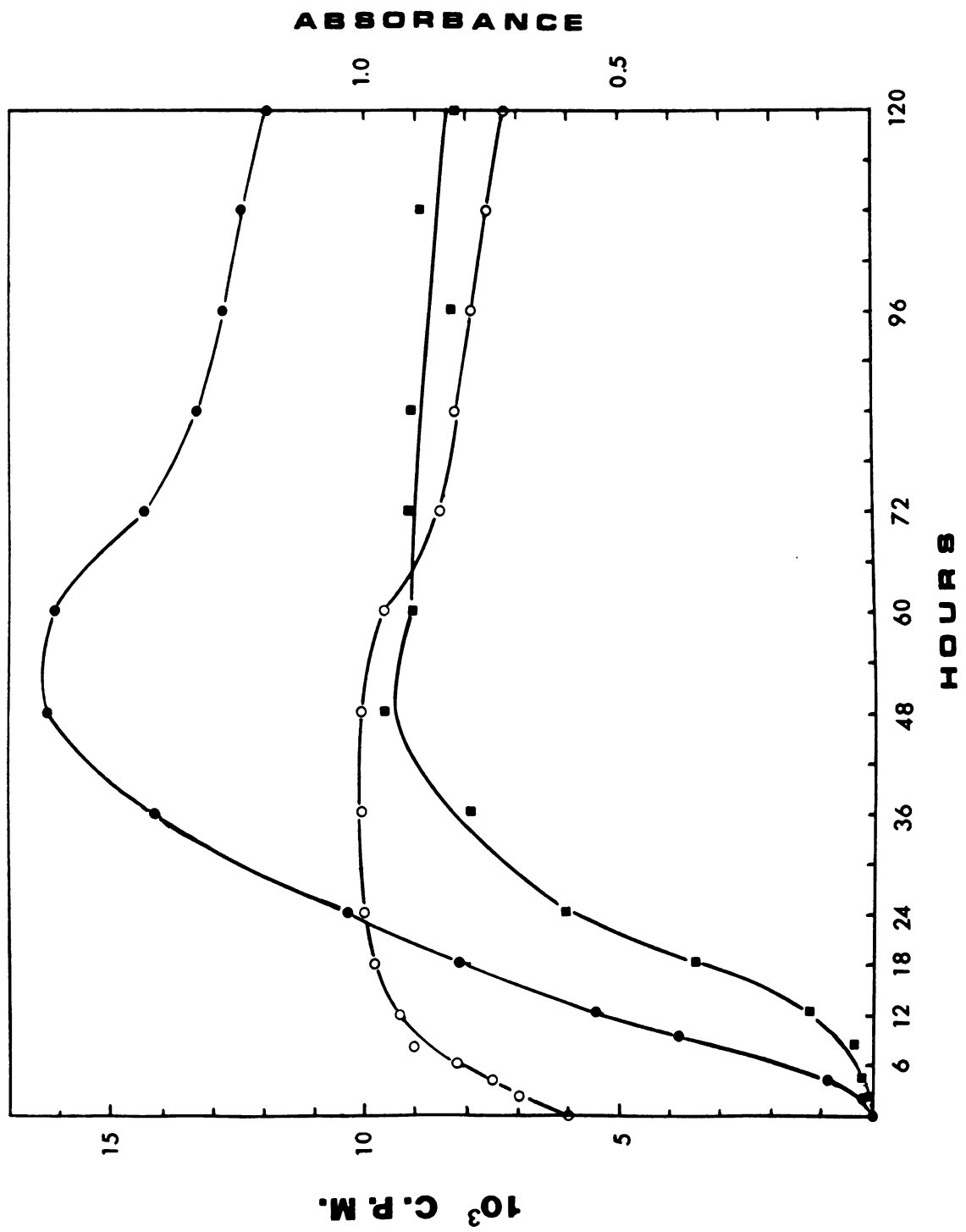
Designated Name	Chemical Structure	Molecular Weight	Melting Point	UV Absorption Peak	Mass Spectrum Base Peak	Vanillin Test
AR ₁		404 and 432 87:13	90-92°C	275 log ε=3.25 281 log ε=3.24	124	(+)
AR ₂		584 and 612 86:14	100-102°C	279-281 log ε=3.20	124	(+)
AR ₃		462 and 490 1:1	95-97°C	265 log ε=3.99 300 log ε=3.59	182	(+)
AR ₄		420 and 488 3:4	95-101°C	281 log ε=3.199 275 log ε=3.19	124	(+)
AR ₅		446 and 474 6:4	94-95°C	271 log ε=3.935 305 log ε=3.57	124	(+)
AP ₁		406 and 434 2:5	107-108 (406) 111-112 (434)	283 log ε=4.09	126	(-)
AP ₂		448 and 476 1:1	97-101	286 log ε=3.72	153	(-)



Fig. 10. Incorporation of ^{14}C -BHB into the lipid fraction during encystment of A. vinelandii.

The change of optical density $\text{O} \longrightarrow \text{O}$, the uptake of ^{14}C -BHB $\bullet \longrightarrow \bullet$ and the radioactive BHB incorporated into the lipid fraction of cells $\blacksquare \longrightarrow \blacksquare$ during the encystment of A. vinelandii. 9 μCi of ^{14}C -BHB was added to 300 ml of cells in Burk's buffer. The radioactivity was expressed as count per minute/ml of culture.





turbidity remained constant for 36 hours and then started to decrease, probably due to the mobilization of PHB with a resultant decrease of refractility of cells.

BHB uptake started at the induction of encystment and increased linearly for 48 hours. At this time, the total radioactivity in the cells amounted to 30% of total radioactivity added to the medium. A gradual decrease of radioactivity then occurred which coincided with the decrease of turbidity.

A significant increase of the radioactivity in the total lipid began at 8 hours after the induction of encystment. It then increased sharply after 12 hours and continued to increase up to 48 hours after induction, after which time it remained relatively constant during the maturation period of cysts. Five days after the induction of encystment, the total lipid fraction contained 68% of the total radioactivity in the cysts.

The possibility that some of the carbon skeleton in these lipids was derived from sources other than BHB was also studied. Vegetative cells were labeled with 2-¹⁴C-glucose and then induced to encyst with non-radioactive BHB. The total radioactivity of cells before and after encystment was determined. These cells had incorporated 10% of the ¹⁴C-glucose added to the medium. Five days after the induction of encystment, 75% of the radioactivity from vegetative cells remained in the cysts and, of this, 14%



was in the lipid fraction. The lipid fraction was separated by TLC and the radioactivity of each compound was counted. The phospholipid fraction contained 45% of the total radioactivity in the lipid or 4.7% of the radioactivity of vegetative cells (RVC). In the lipid compounds synthesized during the encystment, only AR₁ had a significant amount of radioactivity (2.9% RVC). Other compounds only had counts slightly above background, AR₃ + AR₅ (0.8% RVC), AP₁ (0.6% RVC) and AP₂ (0.2% RVC). The radioactivity in these compounds could have been derived from radioactive PHB accumulated during vegetative growth (87). I concluded that these unique lipids were synthesized from BHB used to induce the encystment.

Relative Abundance of These Compounds
in Cyst Lipid

The relative amounts of each component in the lipids of 5 day old cysts were estimated by labeling these compounds with ¹⁴C-BHB during encystment, separating them on TLC, and then measuring the radioactivity in each compound.. The results are shown in Table 2 (arranged according to the R_f value in solvent II). Table 2 compositions of lipids

Total counts in these 9 compounds account for 81% of total counts in the extractable lipid fraction. The rest of the radioactivity carbon counts was recovered mostly in the phospholipid fraction.

Table 2.--Composition of Lipids from 5 Day Old Cysts.

Compound Name	AR ₃	AR ₅	AR ₁	AR ₄	AP ₁	AP ₂	AP ₃	AP ₄	AR ₂	Total % of Count
Percentage of Count in Total Lipid	0.6%	1.84%	28.6%	2.56%	18.15%	9.2%	1.9%	0.7%	17.56%	81%

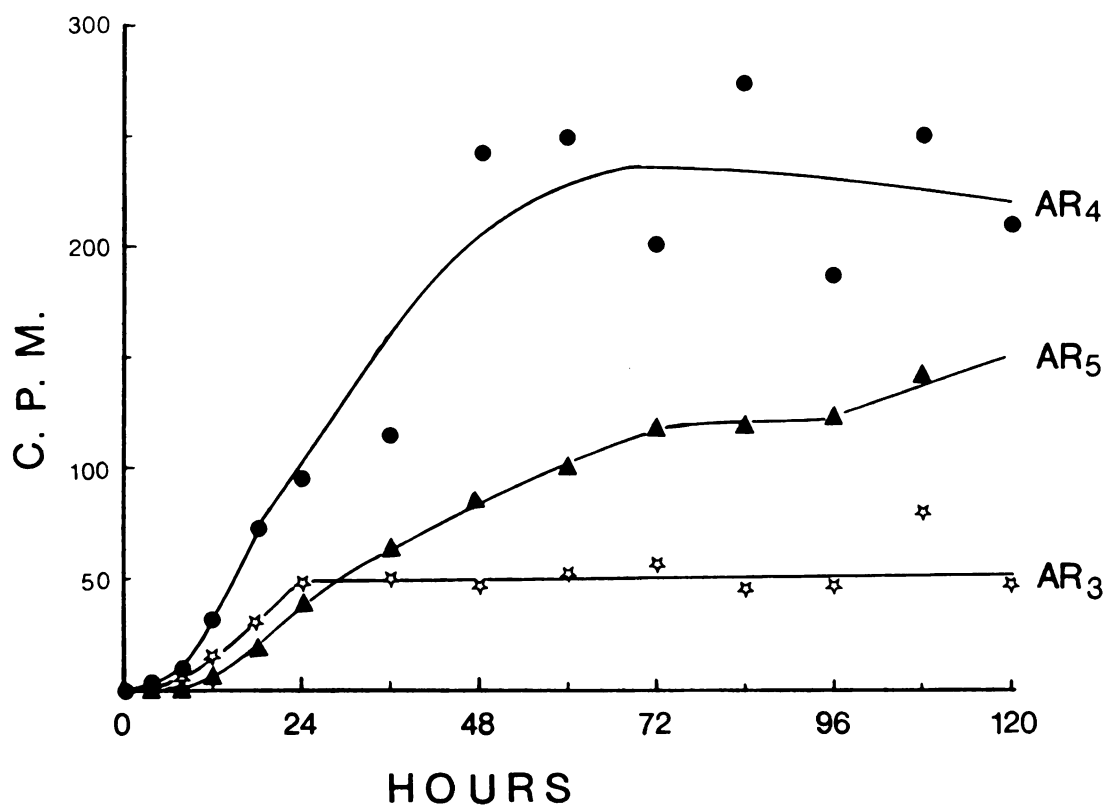
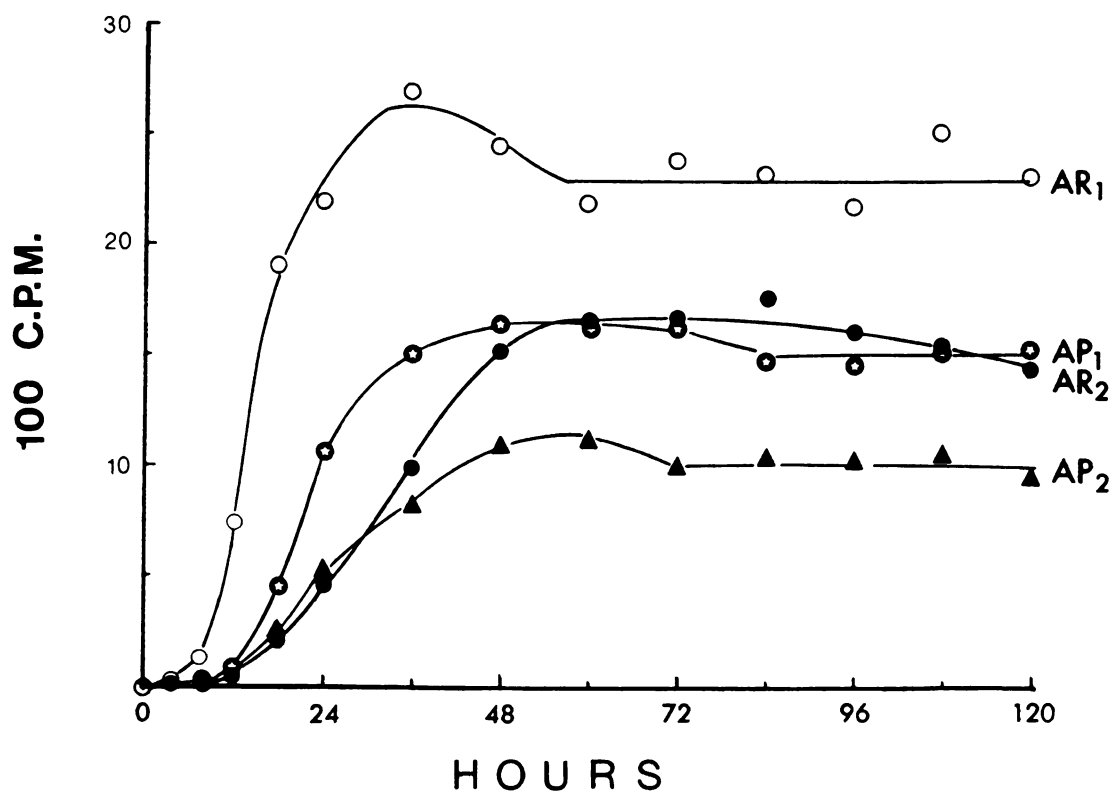
Biosynthesis of Unique Lipids
During Encystment

The time course for the biosynthesis of these compounds during the encystment was followed by inducing encystment with 3-¹⁴C-BHB, sampling at various times and extracting the lipids. The lipids were fractionated on TLC and the radioactivity in each component counted. The results are shown in Fig. 11. The biosynthesis of AR₁ started at about 8 hours after induction of encystment and increased sharply until it reached a peak at 36 hours. A small decrease in the level of AR₁ occurred in the period 36 to 48 hours after which time it remained relatively constant up to 120 hours. Significant synthesis of AR₂, AR₃, AR₄, AR₅, AP₁ and AP₂ was observed 12 hours after encystment. AR₃ reached a plateau of concentration at 24 hours after encystment and stayed at a constant level for the rest of encystment process. This compound, although low in concentration in the developing cyst, was the first of the unique lipids to reach a plateau or steady state level. AP₁ and AP₂ reached their concentration peaks 48 hours after encystment and thereafter decreased somewhat. The amount of AR₂ reached a peak at 60 hours, remained at its high level for about 2 days and then decreased slightly. The biosynthesis of AR₄ was difficult to estimate and subject to large fluctuations because it migrated close to AR₁ during chromatography. A slight contamination with AR₁ could result in a large error in estimating the relative

Fig. 11. The biosynthesis of unique lipids during the encystment of A. vinelandii.

^{14}C -BHB labeled at C-3 position was used to monitor the biosynthesis of these unique lipids. Encystment was induced with 0.2% BHB plus 9 μCi of ^{14}C labeled BHB in 300 ml of cell suspension. Samples were taken at various times during the encystment process and the lipid extracted and separated on TLC. The radioactivity incorporated into each component was determined and expressed as counts per minute/ml of culture.

Top ○—○ AR₁, ●—● AR₂, ☆—☆ AP₁, ▲—▲ AP₂
 Bottom ●—● AR₄, ▲—▲ AR₅, ☆—☆ AR₃





amount of AR₄. Nevertheless, the time course of the biosynthesis of this compound was estimated. AR₄ reached its peak concentration about 48 hours after initiation of encystment and decreased slightly as the cyst matured. A significant increase of AR₅ synthesis occurred 12 hours after encystment and the compound continued to increase in concentration until 72 hours, after which time its rate of synthesis decreased. AR₅ was the only lipid that was still being synthesized after the uptake of BHB had stopped. Except for AR₃ and AR₅, the synthesis of these unique lipids was parallel to the BHB uptake and only a slight decrease occurred in concentration of any of them after they reached their plateau concentration. It is unknown if these compounds were being degraded or simply released into the medium.

Distribution of Unique Lipids in the Cysts

The distribution of unique lipids in the cysts was studied by inducing encystment with ¹⁴C-BHB, fractionating cysts into exine, intine and central bodies, and extracting and analyzing the lipid in each fraction. Central bodies contained 70% of the extractable radioactive lipids, 23.2% were found in the exine and 6.8% in intine (using the total extractable lipid count from all 3 fractions as 100%). The amount in central bodies could have been slightly overestimated because some unbroken cysts were co-precipitated with the central body fraction during its collection by

centrifugation. A few exine fragments were also observed microscopically in this fraction. On the other hand, while a few central bodies were also observed microscopically in the exine fraction, the overall cross-contamination was not severe.

The lipid composition in each fraction is shown in Table 3 and the distribution of each component in the cyst is shown in Table 4. AR_1 and AR_2 were the most abundant components accounting for 35% and 20% respectively of the total lipid in all three fractions. Central bodies seemed to contain a slightly higher percentage of AR_1 whereas AR_2 was uniformly distributed. Of the pyrone derivatives, AP_1 and AP_2 were located principally in the central body fraction which contains 87.47% of total AP_1 and 82.97% of total AP_2 . AR_5 occurred primarily in the exine and intine.

Fates of Unique Lipids During Germination

The fates of the unique lipids were investigated by labeling them with ^{14}C -BHB during encystment, germinating the cysts with glucose, and analyzing the unique lipid distribution at various times during the germination. The results of this experiment are shown in Fig. 12. None of the phenolic compounds (AR_1 , AR_2 , AR_3 , AR_4 or AR_5) underwent appreciable changes in concentration during germination of cysts. However, there was a decrease in pyronic compounds (AP_1 , AP_2 , AP_3 and AP_4) during the outgrowth period. AP_1 decreased by 70% and AP_2 by 60% during this period. Since



Table 3.--The Relative Composition of Unique Lipids in Each Fraction of Cysts.^{a,b,c,d}

Fraction Compound	Exine	Intine	Central Bodies
	Percentage		
AR ₃ and above	13.1	15.7	11.1
AR ₅	9.7	11.8	4.1
AR ₁	33.7	36.1	36.5
AR ₄	3.8	5.3	2.7
AP ₁	7.5	2.2	15.6
AP ₂	5.3	2.6	7.9
AP ₃ and AP ₄	4.0	5.6	2.9
AR ₂	22.9	20.7	19.2

^aThe percentages were calculated using the total radioactive counts from 8 compounds in each structure as 100%.

^bSome neutral lipid co-chromatographed with AR₃ and was not further separated.

^cCompounds are listed according to the sequence of migration distance on TLC.

^dThe distribution of total lipid count in 3 cyst structures: exine 23.2%, intine 6.8%, and central bodies 70.0%.

Table 4.--The Distribution of Unique Lipids in Cysts.^{a,b,c,d}

Fraction	Exine	Intine	Central Bodies
Compound	Percentage		
AR ₃ and above	23.2	6.4	70.4
AR ₅	35.7	10.1	54.2
AR ₁	19.5	4.9	75.6
AR ₄	25.6	8.3	66.1
AP ₁	11.7	0.8	87.5
AP ₂	15.3	1.7	83.0
AP ₃ and AP ₄	25.8	8.4	65.8
AR ₂	23.7	5.0	71.3

^aThe percentages were calculated using the total radioactive counts of each compound in these three structures as 100%.

^bSome neutral lipids co-chromatographed with AR₃ and was not further separated.

^cCompounds are listed according to the sequence of migration distance on TLC.

^dThe distribution of total lipid count in 3 cyst structures: exine 23.2%, intine 6.8%, and central bodies 70.0%.

Table 4.--The Distribution of Unique Lipids in Cysts.^{a,b,c,d}

Fraction Compound	Exine	Intine	Central Bodies
	Percentage		
AR ₃ and above	23.2	6.4	70.4
AR ₅	35.7	10.1	54.2
AR ₁	19.5	4.9	75.6
AR ₄	25.6	8.3	66.1
AP ₁	11.7	0.8	87.5
AP ₂	15.3	1.7	83.0
AP ₃ and AP ₄	25.8	8.4	65.8
AR ₂	23.7	5.0	71.3

^aThe percentages were calculated using the total radioactive counts of each compound in these three structures as 100%.

^bSome neutral lipids co-chromatographed with AR₃ and was not further separated.

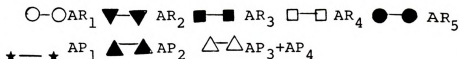
^cCompounds are listed according to the sequence of migration distance on TLC.

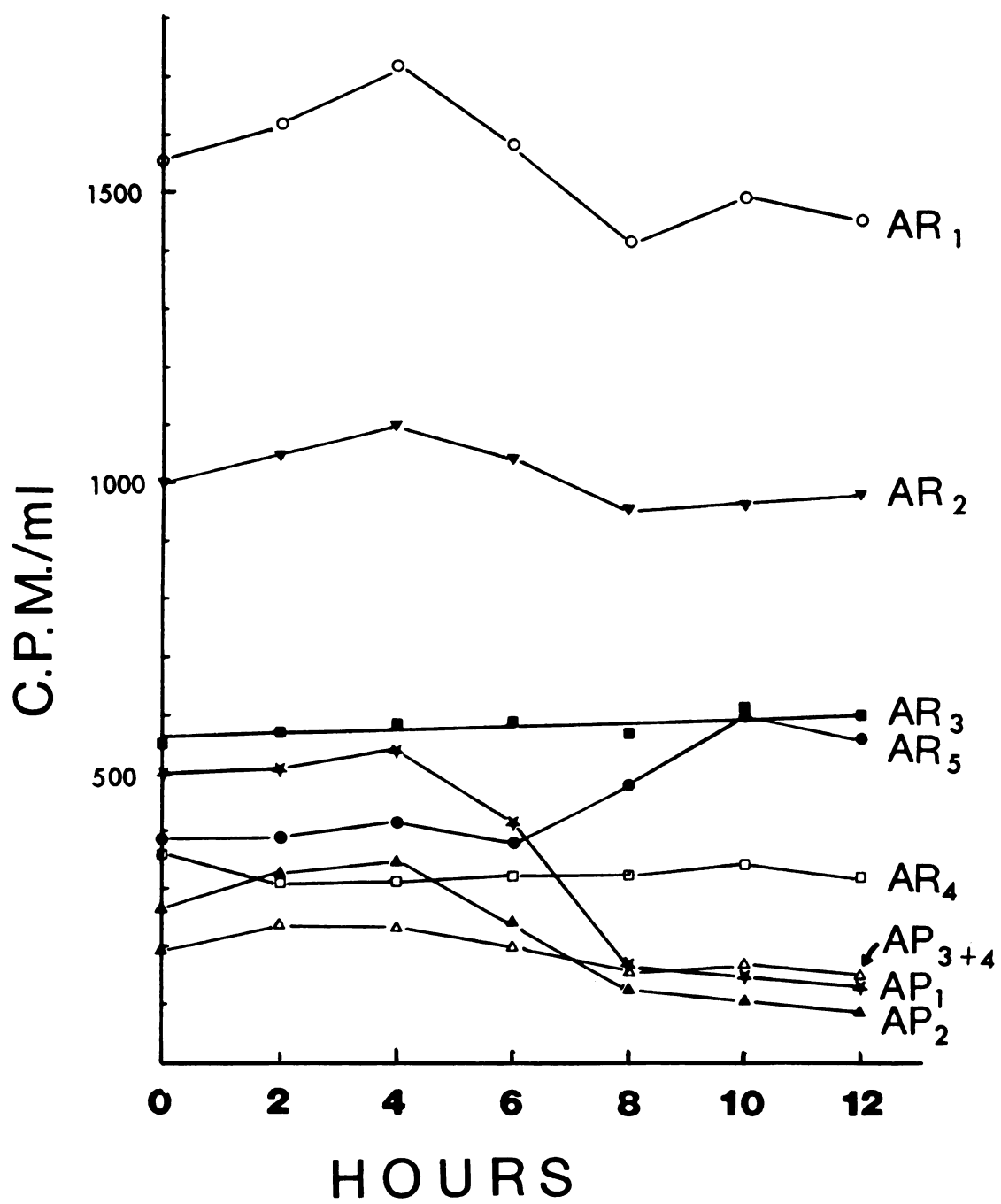
^dThe distribution of total lipid count in 3 cyst structures: exine 23.2%, intine 6.8%, and central bodies 70.0%.



Fig. 12. Fates of unique lipids during the germination of A. vinelandii cysts.

The unique lipids in the cyst were labeled by the addition of 200 mg BHB plus 10 μ Ci of C^{14} -3-BHB into 100 ml of vegetative cell suspension of A. vinelandii. After 5 days of incubation, cysts were harvested, washed twice and suspended in the same volume of Burk's buffer. The cyst germination was started by the addition of 2 ml 50% glucose into the suspension and samples were taken at 2 hour interval. Cells were harvested by the centrifugation and lipid extracted and separated on TLC. The radioactivity in each component of unique lipid was determined and expressed as counts per minute/ml.





these compounds are located predominately in central body, they might play some role in the germination of A. vinelandii. AP₃ and AP₄ also decreased slightly (34%) during outgrowth period (4-8 hours).

The composition of total lipids in this experiment does not reflect the true lipid composition of the developing vegetative cells because the exines which had separated from the germinating cysts were co-sedimented with newly formed vegetative cells during centrifugation. It is not known if the outgrowing vegetative cells contain any of these unique lipids.

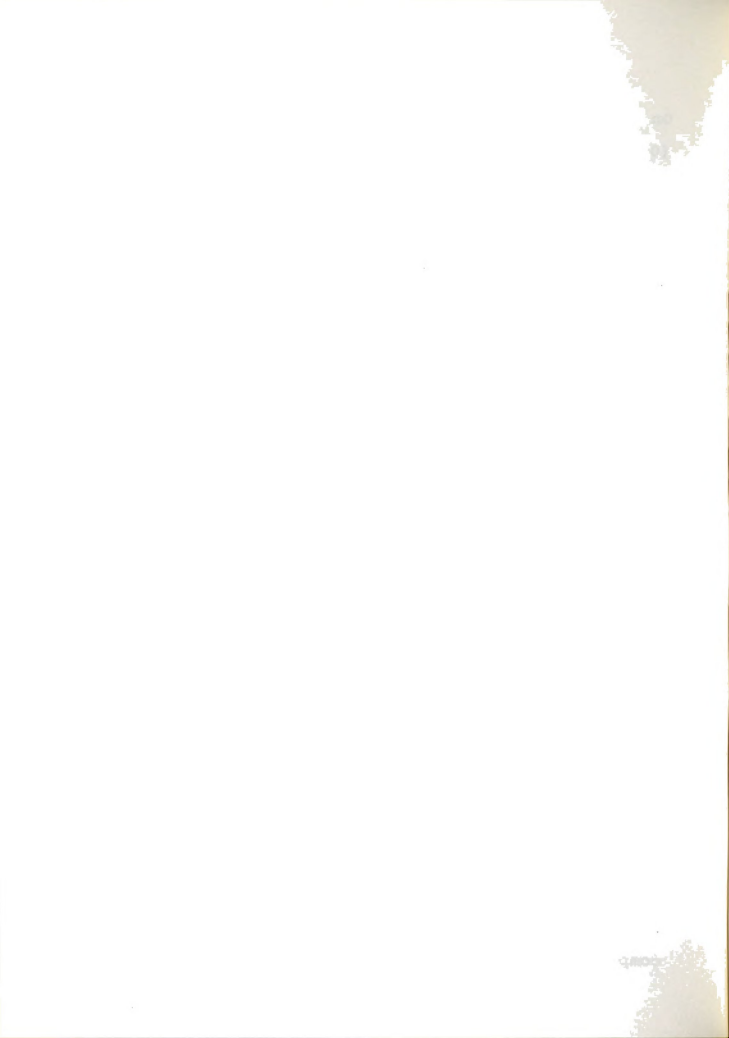
Possible Biosynthetic Pathway of the Unique Lipids

The possibility that the aromatic ring of these compounds is synthesized from an aromatic amino acid biosynthesis pathway (shikimic acid pathway) or derived directly from aromatic amino acids was investigated by the addition of ¹⁴C-labeled phenylalanine to the medium during the encystment. The resulting cysts were labeled by the phenylalanine but only a very small amount of radioactivity was present in the "glyco-" lipid fraction. These results suggest that the aromatic ring of these lipids was not derived from aromatic acids or synthesized via the above mentioned pathway. By growing vegetative cells in ¹⁴C-glucose and then inducing them to encyst with cold BHB, I ruled out the possibility that the long chain alkyl part of these compounds was derived from pre-synthesized fatty acids.

On the other hand, when small amounts of ^{14}C -labeled acetate (0.025% to avoid disturbing the encystment process) were added along with nonradioactive BHB during the encystment, the "glycolipid" fraction was highly labeled, indicating that the unique lipids were synthesized de novo from BHB during encystment and that acetate could be the intermediate of the biosynthesis pathway.

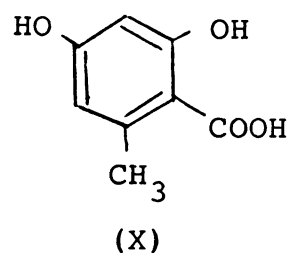
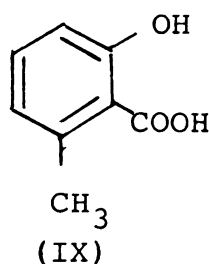
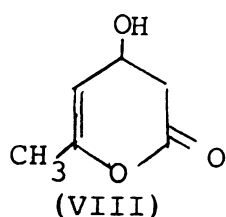
The biosynthetic pathway of many compounds similar to the resorcinols and pyrones identified in this work has been published (9, 23, 28, 102). These related compounds are all synthesized by the condensation of acetate and are called acetogenins. Yalpani et al. (102) found that highly purified fatty acid synthetase of baker's yeast catalyzed the formation of triacetic acid lactone (VIII) from acetyl CoA and malony CoA if NADPH was omitted from the reaction mixture. They proposed that this compound was synthesized by the same mechanism by which fatty acids were being synthesized except for the reduction of β -keto compound by NADPH. The absence of a reducing agent resulted in the formation of an enzyme-bound triacetic acid and the high energy thioester bond between the enzyme protein and the triacetic acid provided energy for the ring closure.

6-Methylsalicylate (IX) is synthesized by the condensation of 1 acetyl CoA plus 3 malony CoA catalyzed by the enzyme "6-methylsalicylic acid synthetase" (23). In Penicillium patulum (22), the synthetase is a multienzyme complex and has properties similar to fatty acid synthetase



(22, 47). Treatment of this enzyme with iodoacetamide converts it into a malonyl CoA decarboxylase, a result which is similar to that obtained on the treatment of fatty acid synthetase with the same reagent. Cerulenin, which inhibits fatty acid synthetase at β -ketoacyl (acyl carrier protein) synthetase step (19), also inhibits the synthesis of 6-methylsalicylate (65).

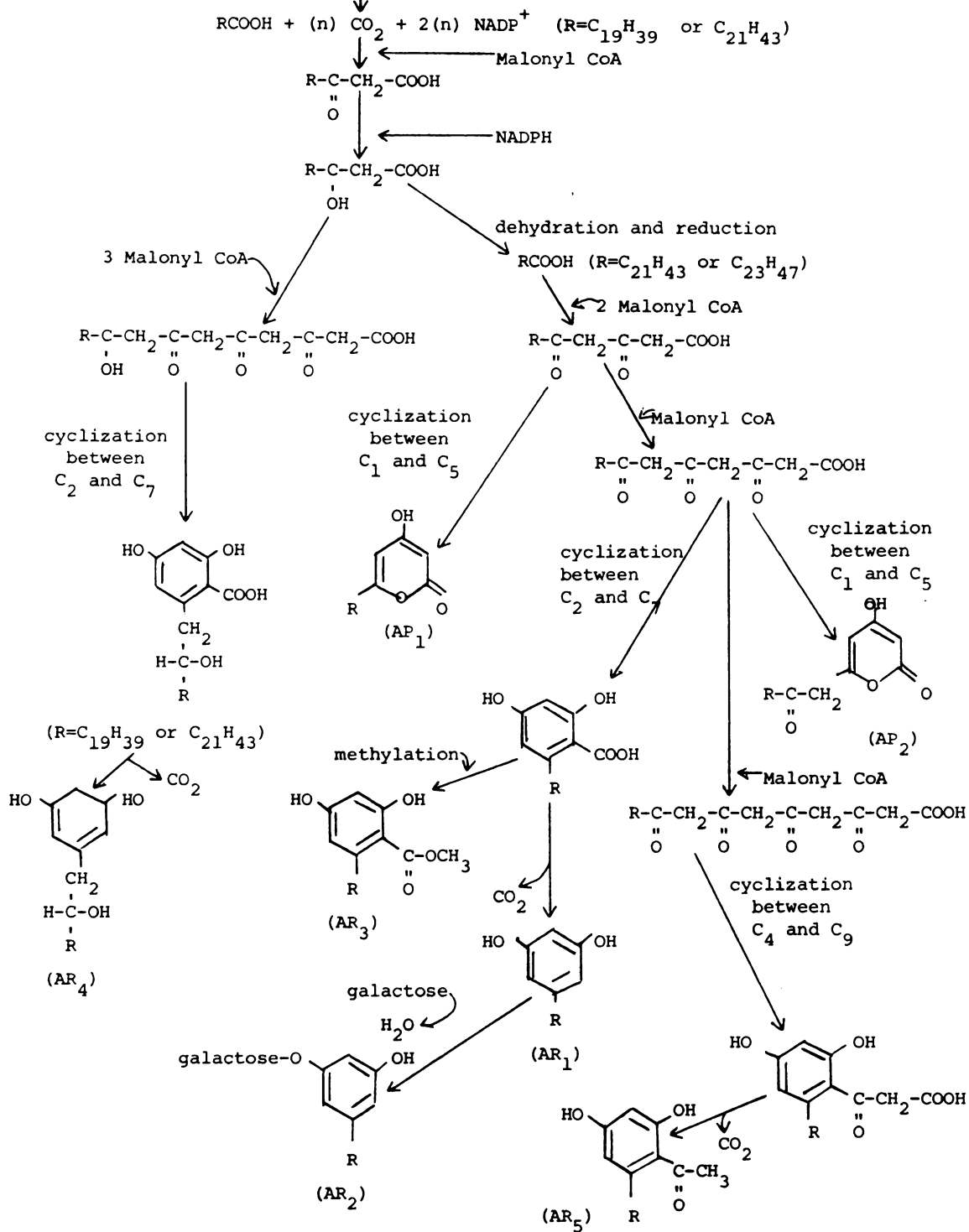
Orsellinic acid (X), which is precursor of many biosynthetic products in molds (4, 5), is also synthesized by the condensation of 1 acetyl CoA and 3 malonyl CoA (28, 61).



The proposed common precursor in the biosynthetic pathway of these aromatic acetogenins is a poly- β -keto (polyketide chain) compound (29, 59). The cyclization of this compound results in the formation of phenolic or pyronic compounds.

I propose the possible biosynthesis pathway of these compounds as in Scheme I. In this pathway, the C_{19} , C_{21} , or C_{23} n-alkyl chain in these lipids is synthesized by the same mechanism as the alkyl chain of a fatty acid. However, when the alkyl chain grows longer (up to 21 or 23 carbons or equivalent to C_{22} or C_{24} fatty acid), the enzyme,



$$\text{Acetyl CoA} + (n) \text{ Malonyl CoA} + 2(n) \text{ NADPH} + 2(n) \text{ H}^+ \quad (n=9 \text{ or } 10)$$


β -ketoacyl-ACP reductase, which carried out the reduction of β -keto group, is no longer functional. At the same time, the enzyme which adds acetyl groups to the chain can still add 2-4 more acetyl groups resulting in the formation of polyketide compounds. Different cyclization mechanisms of these polyketides result in the formation of these unique lipids. In the case of AR₄, the enzyme carried out the acyl group synthesis up to C₂₂ or C₂₄. At this time β -keto group is reduced to an alcohol but the dehydration step fails to occur. Subsequent addition of 3 "acetates" and cyclization results in the formation of AR₄.

These reactions could be carried out by a modified fatty acid synthetase where the activity of enzyme complex is modified (a) during encystment or (b) by the encystment environment where the substrate concentration and co-factor concentration could be significantly different from that which occurs in the vegetative cells. Alternatively, a new enzyme or enzymes could be induced to catalyze these reactions.

Possible Physiological Roles of These Compounds

The physiological roles played by these unique lipids in the cysts is totally unknown but their presence must be significant. This conclusion is drawn from the facts that (a) these compounds account for more than 16% of the total dry weight of Azotobacter cysts and (b) more than one-third of the total BHB taken up by cells during encystment is

used to make these compounds. Structurally, these compounds have long chain hydrophobic "tails" and a hydrophilic "head." These properties are similar to those of phospholipids and hence it is conceivable that these compounds could be incorporated into the cell membrane and alter its properties during encystment. On the other hand, since the exine contains 23% of these lipids, a possible structural role of these compounds also should be considered. These compounds might be able to form a membrane-like structure with their hydrophobic tails inside and the head part of these compounds linked to polyuronic acids to form the sheet-like structure of the cyst exine.

Alternatively, these compounds may only be secondary metabolites of the cell which are produced during the metabolic imbalance occurring in encystment, and, like most alkaloids in plants, their significance to the physiology of the producing organism remains unknown.

Occurrence of Similar Compounds in Nature

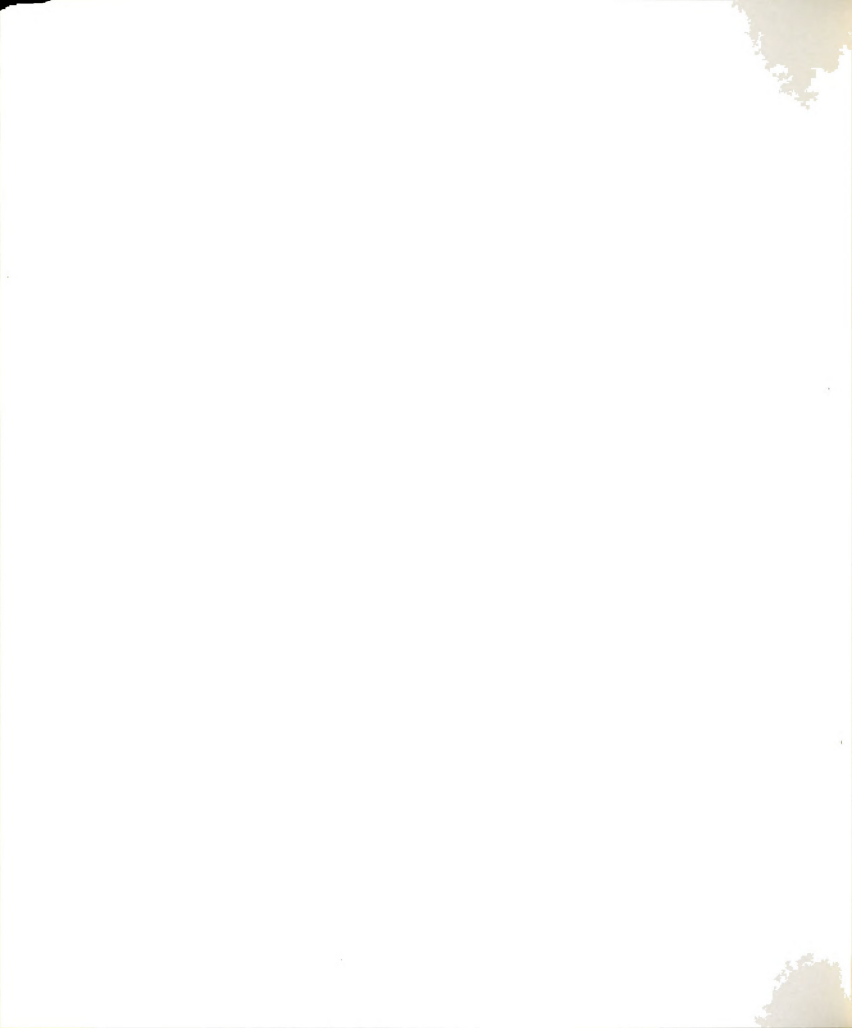
The unique lipids which are synthesized in A. vinelandii during encystment can be divided into 2 categories: phenols and pyrones. The phenols are 5-n-alkyl-resorcinols and their derivatives, 6-n-alkylresorcylic acids and 5-(2-hydroxy-alkyl) resorcinols. Long chain alkylresorcinols have been detected in many species of plants (17, 27, 92, 93). Similar compounds also have been detected in Mycobacterium leprae (12, 15) and Streptomyces species (44).



5-n-heneicosylresorcinol and 5-n-nonadecylresorcinol also have been identified in A. chroococcum (2). 6-methylresorcylic acid (orcillinic acid) have been identified in many fungi including Aspergillus fumigatus, Penicillium cyclopium (71), Chaetomium cochliodes (64), in ethionine treated Penicillium stipitatum, and many others (4). This compound was established as a precursor of many metabolic products in those organisms. I have, however, found no papers in the chemical or biological literature for the occurrence as a natural product of any resorcylic acid with a long chain alkyl group at 6 position or with a β -hydroxyalkyl group.

The pyrones identified in A. vinelandii are 6-n-alkyl-4-hydroxy-pyrone and 6-(2-oxoalkyl)-4-hydroxy-pyrone. Long chain alkyl derivatives of 2,3-dihydropyran-2,4-dione at 6 or 3 and 6 positions have been synthesized (up to eleven carbon chain length) and their bactericidal activity tested (41). Pyrones usually occur as part of large molecules in nature (13, 72). Triacetic lactone (6-methyl-4-hydroxy-2-pyrone) is synthesized by fatty acid synthetase from Bakers yeast when NADPH is not available (102). A metabolite isolated from Penicillium stipitatum (NRRL 1006) was characterized as 3,6-dimethyl-4-hydroxy-2-pyrone (1) and was proposed as a metabolic intermediate leading to the synthesis of other products. Triacetic lactone and tetraacetic lactone (6-(2-oxopropyl)-4-hydroxy-2-pyrone) were isolated from the culture medium of P. stipitatum NRRL 2104 when ethionine was added to the culture to inhibit the

biosynthesis of tropolone (6). These 2 compounds have the same chromophore structure as AP_1 and AP_2 . Tetraacetic lactone was found to be less stable than triacetic lactone and was slowly converted to orsellinic acid and orcinol spontaneously under physiological conditions. Treatment with alkalic accelerates these process. All these descriptions of tetraacetic lactone fit the compound designated AP_2 very well and thus, it is possible that AP_2 convert to AR_1 spontaneously.



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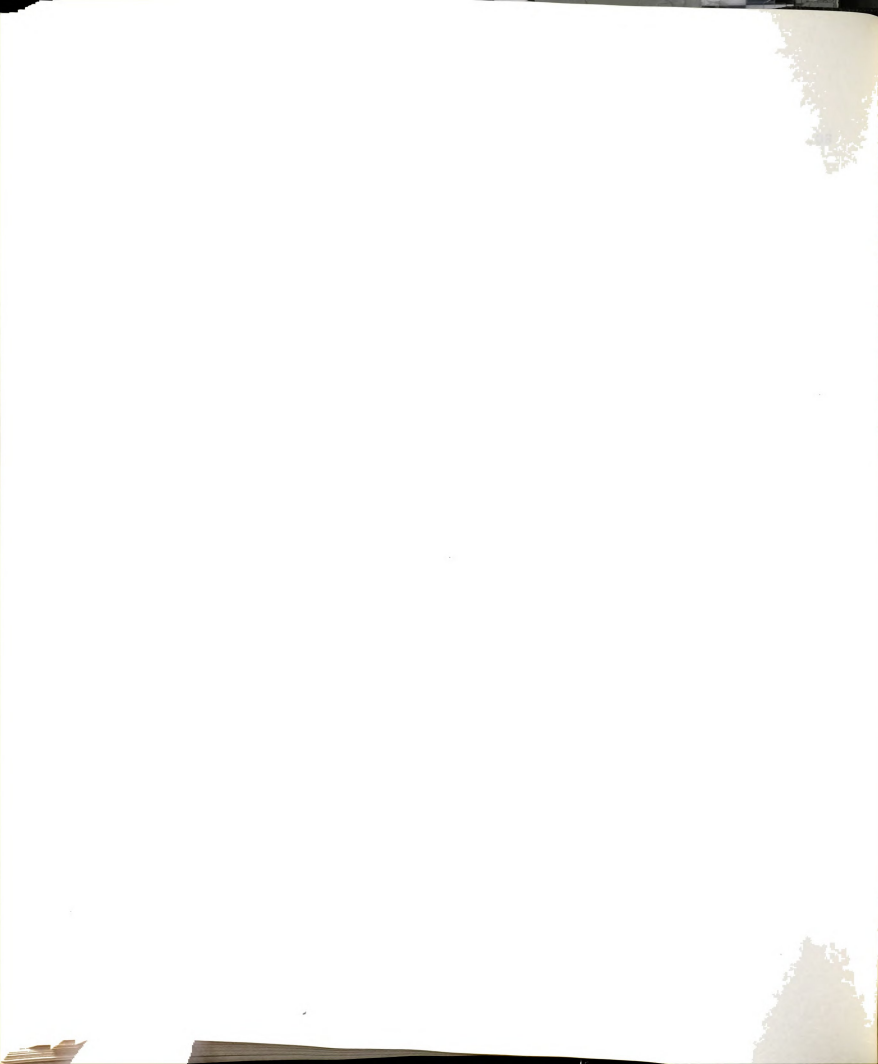


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APPENDIX

APPENDIX

IDENTIFICATION OF C-AMP IN AZOTOBACTER VINELANDII

Introduction

Adenosine 3',5'-cyclic monophosphate (c-AMP) was discovered in animal cells in 1957 (18). Since then, it has been found in all animal cells and has been shown to mediate the effect of a variety of biologically active agents. The occurrence of c-AMP in procaryotic microorganisms was first reported in 1963 by Okabayashi et al. (13) in Brevibacterium liquefaciens and later c-AMP was also detected in Escherichia coli (12). Since then, it has been detected in a wide variety of other microorganisms (20).

C-AMP plays an important role in the regulation of protein synthesis in microorganisms (20). Its control of β -galactosidase induction in E. coli is perhaps, the best example of how c-AMP controls gene expression (5). Beside protein synthesis, c-AMP also affects other cellular phenomena such as the induction of competence for DNA transformation (14, 25), chemotaxis (4) and differentiation (24).

Cell differentiation is the description of events occurring to transform one form of cell to another distinct

type during the life cycle of a particular organism. The process involves the programmed derepression of some particular genes that are specific for the formation and maintenance of the new stage of life cycle and repression of other genes that are not needed. Considerable evidence exists in the literature that cyclic nucleotides plays a major role as mediators in regulating growth and differentiation of many cell types (15, 21). I was interested in investigating the possibility that cyclic nucleotides acted as mediators of gene expression at the transcriptional level during the encystment and germination of Azotobacter vinelandii as well as the possibility that other physiological phenomena (e.g.: the control of nitrogen fixation in this organism) was also mediated by cyclic nucleotides.

Although, some investigators believe that c-AMP exists in all types of cells (20), there is evidence against the existence of c-AMP in some microorganisms (8, 22, 23). Thus, the presence of c-AMP in A. vinelandii had to be confirmed before I attempted any research on the action of c-AMP in this organism.

Materials and Methods

Strain and Culture

Azotobacter vinelandii ATCC 12837 was used in this study. Cells were grown in Burk's nitrogen free medium with either 1% glucose, 1% acetate or 1% glycerol as the sole carbon and energy source. In some cultures, 10 mM of

1000

1000

$(\text{NH}_4)_2\text{SO}_4$ was added as the nitrogen source. Cultures were incubated at 30°C with shaking. The growth of the cultures was monitored by measuring the absorption of light at 620 nm (OD 620) in a spectronic 20 spectrophotometer (Bausch and Lomb).

C-AMP Assay

The protein binding assay for c-AMP (6) was used. Commercial test kits were purchased from Boehringer-Mannheim Biochemical Co. and assays were carried out according to their instructions.

Preparation of Samples for c-AMP Assay

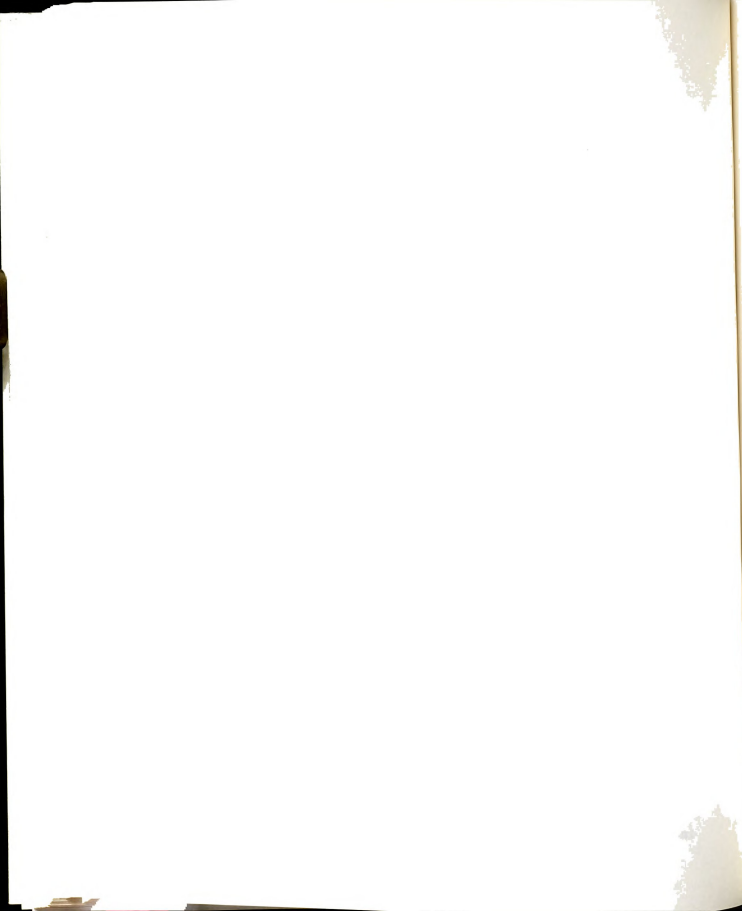
Samples (usually 5 ml) were filtered through double layered, H_2O -washed glass fiber filters (GF/C, 2.4 cm diameter Whatman). The filtrate was collected in a test tube and adjusted to pH 4 with 25% acetic acid and incubated at 95°C for 10 minutes prior to the c-AMP assay. Cells on the filter were washed once with 5 ml of prewarmed Burk's medium and then extracted in 2.5 ml of 0.1 N HCl in 95°C for 10 minutes. The solids in the extract were removed by centrifugation or by filtration through a milipore filter (0.45 μ) that had been boiled in 3 changes of double distilled H_2O . Cell extracts were dried in an evaporator under reduced pressure and redissolved in small amount of distilled H_2O before assay. Five ml of uninoculated Burk's buffer was filtered and the filtrate and solid on the filter were extracted as above and used as controls.

Identification of c-AMP

Two liters of an A. vinelandii culture was grown on 1% glucose until it reached an O.D.=1. Cells were harvested by centrifugation suspended in 100 ml of 0.1 N HCl, and incubated at 95°C for 10 minutes to extract c-AMP. The c-AMP in the supernatant culture fluids and cell extracts were partially purified by the method described by Epstein et al. (5), dried and dissolved in small amount of H₂O. C-AMP in the partially purified samples was separated from other small molecules by thin-layer chromatography. Pre-coated cellulose (Cel 300, 0.1 mm) or silica gel plates (0.25 mm) with or without fluorescence indicator UV₂₅₄ (Macherey-Nagel + Co) were used and the chromatograms developed in solvent (a) (n-butanol 5, glacial acetic acid, 2, water 2 v/v/v/) or (b) (isopropanol 7, concentrated ammonia [15M] 1, water 2 v/v/v). The plates had been pre-washed by developing them in the solvent once and drying them before use. Nucleotides, nucleosides and nucleic acids used as standards were purchased from Sigma Chemical Co.

3',5'Cyclic Nucleotide Phosphodiesterase Treatment

Phosphodiesterase 3'5'-nucleotide from beef heart was purchased from Sigma Chemical Co. Partially purified c-AMP samples from cells and supernatant culture fluid corresponding to 200 ml of the original culture were dissolved in 0.2 ml H₂O. The hydrolysis of cyclic nucleotides in this sample was accomplished as follows. The solution



contained 1.8 μ mole of MgSO_4 , 36 μ mole of Tris-HCl buffer pH 7.5 and about 0.1 unit of phosphodiesterase in a total volume of 1 ml. The mixture was incubated at 30°C for 2 hours. The reaction were stopped by heating the solution in a boiling water bath for 3 minutes. A parallel set of samples was incubated, phosphodiesterase added at the end of incubation period, and the enzyme was denatured immediately in a boiling water bath.

Protein Assay

Two ml of culture were removed, cooled in ice bath and 0.5 ml of 25% cold TCA was added. The resulting precipitate was spun down and the supernatant fluid was removed. Two ml of 0.2 N NaOH was added to the precipitated cells and the mixture was incubated at 100°C for 30 minutes with occasional shaking. The tubes were capped with marbles to prevent evaporation during the incubation. After incubation, the solution was cooled and the undissolved cell debris was removed by centrifugation and supernatant fluid was used for a protein determination. Bovine serum albumin treated the same way was used as a standard. The protein assay was carried out according to the method of Lowry et al. (11).

Results

In the initial attempt to identify c-AMP in A. vinelandii, cells were grown in the following 5 kinds of media: Burk's buffer plus (a) 1% glucose, (b) 1% glucose plus 10 mM $(\text{NH}_4)_2\text{SO}_4$, (c) 1% acetate, (d) 1% acetate plus

10 mM $(\text{NH}_4)_2\text{SO}_4$, and (e) 1% glycerol. Samples were taken when the culture was in mid-exponential growth (O.D. = 0.6-0.8) and the c-AMP level in the cells and supernatant medium was determined. The results are shown in Table A-1.

The results shows that cells grown in acetate seem to release much of their c-AMP into the medium and that cells grown in glycerol have the highest intracellular c-AMP levels. However, the cells grown in glycerol have a very slow growth rate and produce a lot of extracellular polysaccharide. There was no significant difference in the intracellular c-AMP levels between cells grown in glucose, glucose + NH_4^+ , or acetate. Cells grown in acetate + NH_4^+ have very low intracellular c-AMP levels.

Because of the high blank value and the low specificity of the binding assay (6), I felt a more positive identification of c-AMP in this organism was needed. Therefore, c-AMP was partially purified from a culture growing exponentially in 1% glucose and then concentrated. Samples from the medium and the cells were spotted on TLC plates and developed in solvents A and B. The fluorescence indicator increased the sensitivity of nucleotide detection. In both cells and supernatant samples, one major spot was found with an R_f value slightly higher than c-AMP. It was identified as adenosine and only a trace was found at the R_f value corresponding to c-AMP standard. The identification of c-AMP by TLC was not very conclusive.

Table A-1.--Cyclic-AMP levels in A. vinelandii Cells and Culture Fluids.

Growth Medium	Glucose	Glucose+NH ₄	Acetate	Acetate+NH ₄	Glycerol
OD harvested	0.71	0.68	0.75	0.75	0.63
Protein/ml	320 ug	230 ug	215 ug	370 ug	230 ug
c-AMP (pmole/ml) Supernatant	3.2	9.8	35.5	22.3	7.5
Cells	3.8	2.5	2.9	0.8	23.5
c-AMP (pmole/mg protein)	10	42.6	165	60.3	32.6
Cells	11.9	10	13.5	2.13	102.2

Sterile Burk's buffer blank had a value of 0.5 pmole in filtrate and 0.35 pmole in filter extract. This value was subtracted from the assay value.

I then decided to assay for the substance in the partially purified samples that was competing with c-AMP in the binding assay and which was also sensitive to 3',5' cyclic nucleotide phosphodiesterase. The results are shown in Table A-2.

From the table, it can be seen that cells contain 0.2 pmole c-AMP/ml of culture and the medium contained 3.2 pmole c-AMP/ml. Setlow has obtained 50% recovery of c-AMP (23) using this purification procedure. Taking this factor into account, A. vinelandii culture contained 0.4 pmole c-AMP/ml of cells and 6.4 pmole c-AMP/ml in the supernatant medium. The intracellular c-AMP level was therefore 0.9 pmole/mg of protein.

Discussion

The values of the intracellular and extracellular c-AMP level in A. vinelandii, Table A-1, are in the same range as those reported for E. coli (1, 16). After assaying only the phosphodiesterase-sensitive component, Table A-2, I estimate there are about 0.9 pmole c-AMP/mg protein intracellularly and 6.4 pmole c-AMP/ml in the A. vinelandii culture medium. This value is considerably lower than that obtained by the binding assay alone because of the very high background level in the cellular extract of compounds capable of non-specific binding. The discrepancy could be partially due to different growth conditions, but methods used to extract and assay for c-AMP also affect the values

Table A-2.--Phosphodiesterase-Sensitive c-AMP Levels in Cells and Media.

Amount of Partially Purified Sample (100 Time Concentrated)	c-AMP in Pico Mole		
	Without Treatment	Phospho- diesterase Treated	Difference pmole/ml
Cell Extract	0.1 ml	7.5	2.3
		5.2	0.12
	0.05 ml	6.2	2.2
		4	0.22
Supernatant	0.01 ml	1.2	0
		1.2	0
	0.002 ml	0.68	0.08
		0.6	0.2
Supernatant	0.05 ml	40	34
		6	3.4
	0.01 ml	7.5	6.5
		1	3.25
Supernatant	0.001 ml	0.9	0.34
		0.56	1.7

obtained. There are 20-fold differences in c-AMP concentration in the same organism reported by different researchers (15). I conclude that the protein binding assay for c-AMP is not very specific and requires that controls be run in order to subtract for the background. This is particularly true for estimating intracellular c-AMP levels. It would appear that A. vinelandii has about 1/10 of c-AMP content of E. coli K-12 (1) both intracellularly and extracellularly.

The presence of c-AMP in A. vinelandii has never been shown directly, although there is evidence suggesting the existence of c-AMP in this organism. Reuser and Postma (19) found c-AMP could overcome glucose repression in the induction of C₄-dicarboxylic acid anion translocator and C₆-tricarboxylic acid translocator for the Kreb's-cycle intermediates. Lepo and Wyss (9) found the derepression of nitrogenase was accelerated by c-AMP. More recently, Page and Sadoff (14) found c-AMP induced competence of genetic transformation. Goldenbaum (7) found c-AMP inhibited the encystment process in this organism. Using a protein binding assay, he also obtained some c-AMP values in the culture but he was not able to detect 3',5' cyclic nucleotide phosphodiesterase activity in this organism.

From my experiments, I conclude that c-AMP is synthesized by this organism. Although c-AMP identification with TLC was not very conclusive, the binding assay using 3',5' cyclic monophosphate phosphodiesterase treated sample as control was good evidence for the presence of cyclic

nucleotides. In view of the fact that c-GMP is usually present in quantities far less than c-AMP, most of the phosphodiesterase-sensitive substance could have been c-AMP. A search for adenylyl cyclase activity in this organism is essential and, if it can be detected, will be conclusive evidence for the presence of c-AMP in this organism.

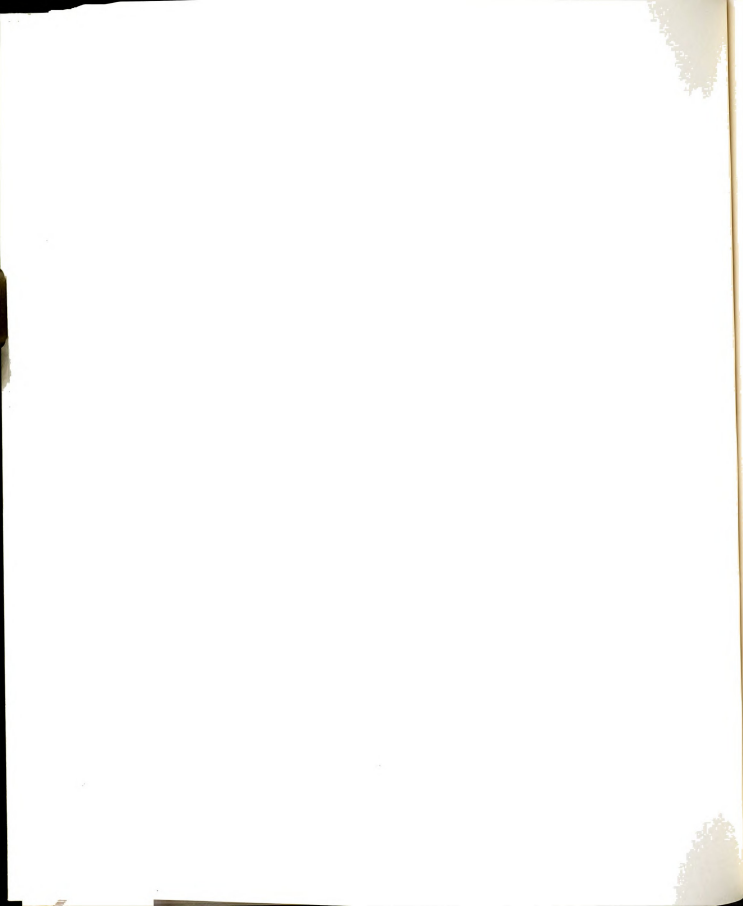
C-AMP has been suggested as a possible mediator during the cell differentiation process in Caulobacter crescentus (24), A. vinelandii (7) and Dictyostelium discoideum (3). Thus it will be of interest to study the changes in intracellular c-AMP levels during encystment and germination in this organism and correlate them with the induction of new enzymes during these processes.

There is also a good possibility that repression and derepression of nitrogenase is mediated by cyclic nucleotides. Prusiner et al. (17) reported that several enzymes involved in ammonia assimilation in E. coli are regulated by c-AMP. Lepo and Wyss (9) found that c-AMP accelerated the derepression of nitrogenase when it was inhibited by NH_4^+ . More recently, Botsford and Villa (2) studied the metabolism of c-AMP in Klebsiella pneumonia and found that the accumulation of c-AMP was not influenced by the cells nitrogen source. They suggested c-AMP was not involved in the regulation of nitrogen fixation in this organism. Lim et al. (10) found the expression of nitrogenase in Rhizobium japonicum was completely inhibited by c-GMP. Hydrogenase and nitrate reductase also were markedly

inhibited by this compound. The intracellular level of c-GMP decreased when nitrogenase was induced but c-AMP had no effect on nitrogenase. It may be that in the regulation of nitrogen fixation, c-GMP plays a more important role than c-AMP.

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